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GENETIC AND PHYSIOLOGICAL STUDIES OF BACILLUS ANTHRACIS RELATED TO DEVELOPMENT OF AN IMPROVED VACCINE

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

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The primary objective of the research is to gain information and develop genetic systems that will contribute to development of an improved vaccine for anthrax. During the year represented by this report our research concentrated largely on (i) the mechanism of transfer of the Bacillus anthracis plasmids, pX01 and pX02, by the conjugative plasmid pX012: (ii) transformation of B. anthracis and B. cereus with plasmid DNA; (iii) transposon mutagenesis in B. anthracis with the transposition selection vector pTV1; (iv) further physical and genetic characterization of phage TP-21, which is active on B. anthracis and whose prophage exists as a plasmid, and exploration of its potential as a vehicle for transposon mutagenesis; (v) further characterization of the conjugative plasmid, pLS20, of B. subtilis and its ability to transfer plasmids among B. anthracis, B. subtilis, B. cereus, and B. thuringiensis.

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Mrs. Virginia M. Miller	(301) 663-7325	SGRD-RMI-S

The self-transmissible plasmid pXO12, originally isolated from strain 4042A of B. thuringiensis subspecies thuringiensis, encodes production of the insecticidal crystal protein. The mechanism of pXO12-mediated plasmid transfer was investigated by following the cotransfer of the tetracycline resistance plasmid pBC16 and the B. anthracis toxin or capsule plasmid, pXO1 or pXO2, respectively. Physical analysis of the transferred plasmids suggested that pBC16 was transferred by the process of donation and the large B. anthracis plasmids were transferred by the process of conduction. The transfer of pXO1 and pXO2 involved the transposition of Tn4430 from pXO12 onto these plasmids.

We have had considerable success in transforming protoplasts of B. cereus. Althoug we were not successful in reproducibly transforming B. anthracis, once we had transformed B. cereus with a plasmid we could then transfer it to B. anthracis by CP-51-mediated transduction. We have also been able to transform cells of B. cereus as well as B. anthracis by electroporation. The electroporation method is much easier and faster than th protoplast transformation method. It will be the method of choice in the future. The fact that we have been able to transform B. cereus and B. anthracis has opened up the possbility of using the temperature-sensitive transposition selection vector pTV1, which contain the MLS resistance transposon Tn917, for transposon mutagenesis in B. anthracis. We have isolated auxotrophic mutants which have Tn917 inserted in the chromosome, and we have also isolated derivatives of plasmid pXO1 which have Tn917 insertions.

We have demonstrated that the phenomenon of transductional shortening can be applie to Tn917-tagged pXO1. Phage CP-51, which can mediate plasmid transduction in B. anthracis, can package DNA about half the size of pXO1. By using strains of B. anthracis carrying pXO1::Tn917 as donors, we have been able to select erythromycin-resistant transductants which contain derivatives of pXO1 about half as large as the wild-type plasmid. We are currently testing the strain carrying the shortened version of pXO1 for phenotypic changes. If Tn917 inserts randomly in pXO1, it should be possible to isolate a large variety of shortened pXO1 plasmids. The availability of a collection of deleted plasmids would be of considerable help in studying the biology of pXO1 as it relates to the virulence of B. anthracis and the synthesis of protective antigen and other components of anthrax toxin.

The 29-megadalton plasmid of B. thuringiensis subsp. kurstaki strain HD-1 is the prophage of a phage we have designated TP-21. This appears to be the first phage with a plasmid prophage described for the genus Bacillus. TP-21 mediates both chromosomal and plasmid transduction in B. anthracis, B. cereus, and B. thuringiensis. TP-21 lysogens have been isolated which have transposon Tn917 inserted in the prophage. Although insertion of Tn917 rendered some isolates defective, several isolates carrying this element produced viable phage which conferred erythromycin resistance upon newly lysogenized hosts. Certain mutants of TP-21 which carry Tn917 appear to be temperature sensitive fo replication in B. cereus and unable to replicate at all in B. anthracis. Such mutants a currently being tested as vehicles for transposon mutagenesis in B. anthracis.

B. subtilis (natto) 3335 harbors a plasmid, pLS20, which encodes functions respons ble for conjugal transfer of plasmids among genetically related and genetically distinct species of Bacillus, including B. anthracis. We have isolated derivatives of pLS20 whic contain the transposon Tn917. Investigation of the transposon-tagged derivatives should allow the localization and cloning of the transfer gene(s) encoded by this novel B. subtilis plasmid. Several tagged plasmids that were defective in promoting conjugal transf had Tn917 inserted in a 10.8-kb BglII fragment. Analysis of pLS20 derivatives that have Tn917 inserted in this fragment suggests that the fragment contains at least two loci in volved in conjugation.

SUMMARY

The self-transmissible plasmid pXO12 (112.5 kb), originally isolated from strain 4042A of Bacillus thuringiensis subspecies thuringiensis, codes for production of the insecticidal crystal protein. The mechanism of pXO12-mediated plasmid transfer was investigated by following the cotransfer of the tetracycline resistance plasmid pBC16 and the Bacillus anthracis toxin or capsule plasmid, pXO1 or pXO2, respectively. In matings of B. anthracis donors with B. anthracis and Bacillus cereus recipients, the number of Tc^r transcipients ranged from 4.8×10^4 to 3.9×10^6 per ml (frequencies ranged from 1.6×10^{-4} to 7.1×10^{-2}), and 0.3% to 0.4% of them simultaneously inherited pXO1 or pXO2. Physical analysis of the transferred plasmids suggested that pBC16 was transferred by the process of donation and the large B. anthracis plasmids were transferred by the process of conduction. The transfer of pXO1 and pXO2 involved the transposition of Tn4430 from pXO12 onto these plasmids. DNA-DNA hybridization experiments demonstrated that Tn4430 was located on a 16.0-kb AvaI fragment of pXO12. Examination of Tra^- and Cry^- derivatives of pXO12 showed that this fragment also harbored information involved in crystal formation and was adjacent to a restriction fragment containing DNA sequences carrying information required for conjugal transfer.

During the past year we have had considerable success in transforming protoplasts of Bacillus cereus. Although we were not successful in reproducibly transforming B. anthracis by the same procedure, once we had transformed B. cereus with a plasmid we could then transfer it to B. anthracis by CP-51-mediated transduction. We have also been able to transform cells of B. cereus as well as B. anthracis by electroporation. The electroporation method is much easier and faster than the protoplast transformation method. It will be the method of choice in the future. With both methods the efficiency of transformation decreased dramatically as the size of the plasmid DNA increased. However, it seems likely that we will be able to modify the electroporation procedure and increase the efficiency of transformation. The fact that we have been able to transform B. cereus and B. anthracis has opened up the possbility of using the temperature-sensitive transposition selection vector pTV1, which contain the MLS resistance transposon Tn917, for transposon mutagenesis in B. anthracis. The few experiments we have done thus far on transposon mutagenesis are sufficient to show that Tn917 can be used success-

fully in B. anthracis. We have isolated auxotrophic mutants which have Tn917 inserted in the chromosome and we have also isolated derivatives of plasmid pXO1 which have Tn917 insertions. We are currently in the process of testing strains carrying pXO1::Tn917 for any changes in phenotype.

We have demonstrated that the phenomenon of transductional shortening can be applied to Tn917-tagged pXO1. Phage CP-51, which can mediate plasmid transduction in B. anthracis, can package DNA about half the size of pXO1. By using strains of B. anthracis carrying pXO1::Tn917 as donors, we have been able to select MLS^r transductants which contain derivatives of pXO1 about half as large as the wild-type plasmid. We are currently testing the strain carrying the shortened version of pXO1 for phenotypic changes. If Tn917 inserts randomly in pXO1, it should be possible to isolate a large variety of shortened pXO1 plasmids. The availability of a collection of deleted plasmids would be of considerable help in studying the biology of pXO1 as it relates to the virulence of B. anthracis and the synthesis of protective antigen and other components of anthrax toxin.

We have found that the 29-megadalton plasmid of B. thuringiensis subsp. kurstaki strain HD-1 is the prophage of a phage we have designated TP-21. This appears to be the first phage with a plasmid prophage described for the genus Bacillus. TP-21 mediates both chromosomal and plasmid transduction in B. anthracis, B. cereus, and B. thuringiensis. TP-21 plaque-forming particles contain approximately 49 kilobase pairs of DNA which appears to be circularly permuted and terminally redundant. TP-21 lysogens have been isolated which have the erythromycin resistance transposon Tn917 inserted in the prophage. Although insertion of Tn917 rendered some isolates defective, several isolates carrying this element produced viable phage which conferred erythromycin resistance upon newly lysogenized hosts. Results of tests with TP-21::Tn917 demonstrated a broad host range among B. anthracis, B. cereus and B. thuringiensis. Certain mutants of TP-21 which carry Tn917 appear to be temperature sensitive for replication in B. cereus and unable to replicate at all in B. anthracis. It seems that such mutants should be useful as vehicles for transposon mutagenesis. Experiments are currently being carried out to investigate this possibility.

Bacillus subtilis (natto) 3335 harbors a plasmid, pLS20, which encodes functions responsible for conjugal transfer of plasmids among genetically related and genetically distinct species of Bacillus. The use of several re-

striction endonucleases has revealed the size of pLS20 to be 64 kb. This is an increase of approximately 9 kilobases over the original estimate of 55 kb. The transfer genes of pLS20 appear to be unique, as they exhibited no homology with the transfer genes of pXO12 or pAMβ1, as determined by DNA-DNA hybridization experiments.

Utilization of the transposition selection vector pTV1 allowed the isolation of pLS20-derived plasmids which contain the transposon Tn*917*. Investigation of the transposon-tagged derivatives should allow the localization and cloning of the transfer gene(s) encoded by this novel *B. subtilis* plasmid. Several tagged plasmids that were defective in promoting conjugal transfer had Tn*917* inserted in a 10.8-kb *Bgl*II fragment. Results of analysis of pLS20 derivatives that have Tn*917* inserted in this fragment suggest that the fragment contains at least two loci involved in conjugation. The insertion of the transposon into some locations within the fragment abolished self-transfer but did not abolish the ability to mobilize pBC16. This implies that the transposon has inserted within the region of the plasmid that is nicked prior to transfer, i.e., the origin of transfer. Insertion of Tn*917* into other sites within this fragment rendered the host cell completely transfer-deficient. This suggests that the transposon inserted within the gene that encodes the site-specific nuclease required to nick plasmid DNA prior to transfer.

For some of the pLS20::Tn*917* isolates, growth of host strains in the presence of erythromycin generated deletions of approximately 15-20 kb of pLS20 DNA. These deletants were stable, and cells harboring them were not fertile, suggesting that the deletions encompassed one or more genes necessary for plasmid transfer. The DNA deleted in these plasmids included the 10.8-kb *Bgl*II fragment discussed above. This information, combined with that obtained from the plasmids carrying insertions of Tn*917* within the 10.8-kb fragment, provides evidence that this region of pLS20 is required to confer a transfer-proficient phenotype upon the host cell.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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ANNUAL PROGRESS REPORT

This is the third annual report submitted under contract DAMD17-85-C-5212. Research on the contract which began August 1, 1985 is a continuation of research previously carried out under contract DAMD17-80-C-0099.

During the year represented by this annual report our research concentrated largely on (i) the mechanism of transfer of the B. anthracis plasmids, pXO1 and pXO2, by the conjugative plasmid pXO12; (ii) transformation of B. cereus and B. anthracis with plasmid DNA; (iii) transposon mutagenesis in B. anthracis with the transposition selection vector pTV1; (iv) further physical and genetic characterization of phage TP-21, which is active on B. anthracis and whose prophage exists as a plasmid and exploration of its potential as a vehicle for transposon mutagenesis; (v) further characterization of the conjugative plasmid, pLS20, of B. subtilis and its ability to transfer plasmids among B. subtilis, B. cereus, B. thuringiensis, and B. anthracis.

In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. Table 1 lists the bacterial strains, plasmids, and bacteriophages referred to in this report. Escherichia coli strain JM83(pHT44), provided by M.-M. Lecadet, was used as a source of Tn4430 DNA. Plasmid pHT44 contains one copy of Tn4430 deleted by 2 basepairs at each end and was constructed by inserting the internal 4.2-kb KpnI fragment of Tn4430 in the KpnI site of the vector plasmid pUC18 (14). Bacillus subtilis PSL1 was transformed with plasmid pTV1 DNA and the transformant was used as a source of Tn917. The recombinant plasmid pTV1 (31), provided by P. Youngman, is a transposition-selection vector containing the rep functions of pE194, the chloramphenicol resistance determinant (cat gene) of pC194, and the inducible erythromycin resistance transposon Tn917.

Media. For convenience to the reader, compositions of the various culture media referred to in this report are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

$NBYCO_3$ agar: NBY agar with 7 g of $NaHCO_3$.

Phage assay (PA) broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

$PACO_3$ agar: PA agar with 7 g of $NaHCO_3$.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

LG broth: L broth with 1 g of glucose.

BHI broth: Brain heart infusion broth (Difco), 37 g.

Peptone diluent: Peptone (Difco), 10 g. Used for diluting phage and bacterial cells.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal 3: To Minimal 1 were added 10 mg of thiamine hydrocloride and 200 mg of glycine.

Minimal 4NH: To Minimal 3 were added 40 mg of L-methionine and L-histidine, and 10 mg of nicotinamide.

Minimal XO: To Minimal 1 were added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

CA-agarose medium: CA-agarose medium for the detection of colonies producing protective antigen was prepared as follows: 0.75 g of agarose was added to 100 ml of CA broth (prepared as described by Thorne and Belton [28]) and the mixture was steamed until the

agarose was dissolved. When the medium cooled to about 50° C, 1 ml of 20% glucose, 8 ml of 9% $NaHCO_3$, 6 ml of goat antiserum to B. anthracis, and 10 ml of horse serum were added. The medium was dispensed in petri plates (13 ml per plate) and the plates were left with their lids ajar while the agarose solidified. The plates were usable after 1 hr.

Antiserum. B. anthracis antiserum was kindly supplied by personnel of USAMRIID.

Propagation and assay of bacteriophage CP-51. The methods described previously (23, 26) were followed. The indicator for routine assay of these phages was B. cereus 569.

Propagation and assay of phage TP-21 and TP-21c lysates. Cell-free lysates of TP-21 were prepared by growing cultures of lysogens from a loop of cells or spores in 25 ml BHI broth with 0.1% glycerol. Following 16-18 h of incubation at 30°C the cultures were centrifuged to remove the cellular debris and filtered through a 0.45-µm HA Millipore filter. Lysates of TP-21c were prepared by lytic infection of B. cereus 569 in broth cultures. All lysates were confirmed to be free of bacterial contamination by plating samples on L agar.

TP-21 and TP-21c were assayed by the soft agar overlay technique on NBY agar containing 0.1% glycerol, with spores of B. cereus 569 as the indicator. Phage was diluted in 1% peptone and 0.1 ml was mixed with 0.1 ml of indicator in 2 ml of soft agar. Glycerol (0.1 % w/v) was also added to the top agar to improve the ability to detect plaques of phage TP-21. Plates were incubated at 30°C and plaques were counted after 14 h. Exposure of assay lawns to chloroform vapor made the plaques easier to see. This was done by adding chloroform to the lids of inverted assay plates (glass plates) and letting them stand at room temperature for 30 min.

Nitrosoguanidine mutagenesis. Bacteriophage TP-21 was mutagenized by growing lysogens in medium containing N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at a final concentration of 100 µg/ml. A stock solution (1 mg/ml) was prepared by first dissolving 10 mg of NTG in 1 ml of acetone and then adding H_2O to 10 ml final volume. For use , 2.5 ml of this stock was added to 25 ml of BHI broth containing 0.1% glycerol.

Preparation of lysogens. Cell free lysates of TP-21 were assayed on lawns of the strains of which lysogens were desired. The lysogens were ob-

tained from the centers of turbid plaques by stabbing plaques with sterile toothpicks and then streaking for isolation on PA agar plates.

Lysogens of the TP-21 mutants TP-21c-3, TP-21c-7, TP-21c-8 and TP-21c-12 were prepared by streaking an L agar plate with growth from a lawn of cells in soft agar which had undiluted phage lysate spotted onto it. Colonies of presumptive lysogens were picked to both a master plate and to a soft agar lawn of B. cereus 569 spores. Those colonies which made a zone of clearing on the B. cereus lawns were then tested for the presence of the prophage plasmid by agarose gel electrophoresis.

Isolation of phage TP-21 DNA. The phage from a lysate containing 10^{10} or more PFU per ml was collected by centrifugation for 2 h at 25,000 rpm. The phage pellet from each 65 ml of culture was then suspended in 1 ml of SSC buffer and pooled with the other phage suspensions. An equal volume of freshly distilled phenol saturated with SSC was added and the tube was gently rolled for 10 min. The mixture was centrifuged at 10,000 rpm for 30 min. The phenol layer was removed and the aqueous layer was centrifuged at 10,000 rpm for 20 min to pellet the protein. The aqueous phase was poured into a sterile tube and the deproteinization procedure was repeated. The DNA was precipitated by first adding one half volume of 7.5 M ammonium acetate and mixing well. Two volumes of 95% ethanol were added and the tube contents were mixed well by inverting the tube 20 times. The tube was held on ice for 10 min and then the DNA pellet was collected by centrifuging at 0°C for 1 h at 10,000 rpm. The ethanol was decanted and the DNA pellet was allowed to drain over paper towels for 5 to 10 min before resuspending in 5 ml of TES (50 mM Tris-HCl, 50 mM NaCl, 3 mM EDTA [pH 8.0]).

Test for capsule production. The ability of B. anthracis and B. cereus to produce capsules was determined by growing cells on PA agar or NBY agar incubated in air for those mutants which did not require bicarbonate for capsule production, or on $NBYCO_3$ agar or $PACO_3$ agar, each supplemented with 10% (v/v) horse serum, and incubated in 20% CO_2 for those strains which produced capsules only in the presence of bicarbonate. Plates were incubated at 37°C for 24 to 48 h.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (9). Cells were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10 μg/ml). With some strains better results were obtained

when 0.1% (w/v) glycerol was included in the BHI broth to prevent sporulation. Best results with strains of B. anthracis were obtained when the BHI broth was supplemented with 10% (v/v) horse serum. The inoculum for each flask was a loop of growth from an L agar plate which had been streaked with a loop of spores and incubated at 37°C for 16 to 24 hours. Cultures were incubated at 37°C on a rotary shaker (100 to 160 rpm) for 13 to 16 hours. Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and resuspended in 1 ml of E buffer (0.04 M Tris-OH (Sigma), 0.002 M EDTA (tetrasodium salt, Sigma), 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer prepared by adding 3 gm of sodium dodecyl sulfate and 5.0 ml of 3.0 N NaOH to 100 ml of 15% (w/v) sucrose in 0.05 M Tris-OH. The tubes were rapidly inverted 20 times to mix the cells and buffer and they were then held in a 60°C water bath for 30 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C and the aqueous phase was removed for electrophoresis.

The procedure as described above was used for B. anthracis, B. cereus, and B. thuringiensis. It was modified slightly for B. subtilis and B. licheniformis. Cultures were grown for 16 hours in BHI broth supplemented with 0.1% glycerol, or in LG broth for B. subtilis natto. After cell pellets were suspended in 2 ml of E buffer, lysozyme was added to give a final concentration of 2 mg per ml, and suspensions were incubated at 37°C for 45 to 60 minutes. Two ml of lysis buffer as described above were added and tubes were inverted 20 times. After the suspensions were incubated 30 minutes at 60°C they were cooled on ice and 0.5 ml of 2M Tris-OH was added. The tubes were inverted 20 times, and following the addition of 6 ml of cold phenol-chloroform mixture to each tube, they were inverted 20 times again. Finally the tubes were centrifuged at 10,000 rpm for 10 minutes and the aqueous layer was withdrawn.

For electrophoresis of plasmid DNA, extracts (40 µl) were mixed with 10 µl of tracking dye (0.25% bromphenol blue, 15% ficoll) and samples (40 µl) were applied to horizontal 0.7% agarose (Sigma, Type II medium EEO) gels prepared and run in Tris-borate buffer (0.089 M Tris-OH, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3). Electrophoresis was carried out at 70 V for 90

to 120 min at room temperature. Gels were stained with ethidium bromide (1 µg/ml) in Tris-borate buffer).

Method for isolating plasmid DNA suitable for restriction analysis. The above procedure for extracting plasmid DNA was modified in such a way that very little or no chromosomal DNA is present in the preparations. At least there is not enough chromosomal DNA present to interfere with restriction analysis of plasmids. The modified procedure is the same as the procedure described above through the lysis step at 60°C. At that point cell debris and unlysed cells were removed by centrifugation at 10,000 rpm for 15 minutes at 5°C. The supernatant fluid was decanted and placed in an ice bath. Ice-cold 2 M Tris (0.5 ml, pH 7.0) was added to neutralize the lysate and mixing was accomplished by inverting the tubes 20 times. The lysate was extracted with 6 ml of cold phenol-chloroform by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 minutes at 5°C, and the aqueous phase was removed. One-half volume of 7.5 M ammonium acetate was added, and the plasmid DNA was precipitated by adding twice the final volume of ice cold 95% ethanol. The tubes were held on ice for 15 minutes and then centrifuged at 10,000 rpm for 30 to 60 minutes at 5°C. The ethanol was decanted, 5 ml of cold 70% ethanol was added to each tube which was then mixed gently on a vortex mixer, and the DNA was collected by centrifugation at 10,000 rpm for 15 minutes at 5°C. After the ethanol was decanted, the tubes were inverted over paper towels for 10 minutes and then placed in a vacuum desiccator for at least 2 hours to dry the DNA thoroughly. The DNA was dissolved in 0.1 to 0.5 ml of TES (0.05 M Tris-OH, 0.05 M NaCl, 0.005 M EDTA, pH 8.0) containing 50 µg of RNase per ml.

If larger preparations of plasmid DNA are desired, the above procedure can be scaled up successfully.

Plasmid DNA for nick translation was purified further by cesium chloride density gradient centrifugation.

Restriction endonuclease digestions. Restriction endonuclease digestions were carried out under conditions recommended by the supplier of the enzymes. Usually 10 to 20 µl of DNA (1.0 to 1.5 µg) in TES (pH 8.0) was added to 5 to 10 units of enzyme in a 1.5 ml Eppendorf tube. Appropriate amounts of distilled water and 10X buffer were added to give a total volume of 100 µl. Reaction mixtures were incubated in a 37°C water bath for 2 to 15 h. Digests were heated at 65°C for 10 minutes to stop reactions and then resolved on

agarose gels. Molecular weights of DNA fragments were determined by comparing their mobilities to those of a kilobase ladder consisting of fragments ranging in size from 0.2 to 12.2 kb or a set of high molecular weight markers ranging in size from 8.3 to 48.5 kb. Both sets of DNA size standards were obtained from Bethesda Research Laboratories.

Transduction of pXO2. Bacteriophage CP-51ts45 was propagated on B. anthracis and assayed on B. cereus 569. Recipient cells for transduction were grown in 250-ml flasks containing 25 ml of L broth (for B. cereus) or BHI broth with 0.5% glycerol (for B. anthracis) and incubated at 37°C on a rotary shaker at 250 rpm. Cells from a 10% (vol/vol) transfer of a 16-h culture were grown for 5 h. Cells (0.1 ml containing approximately 10^8 CFU) and phage (0.1 ml containing approximately 5 x 10^9 PFU) were spread together on $NBYCO_3$ or $PACO_3$ agar. Plates were incubated at 37°C in 20% CO_2. After 3 h, 0.1 ml of phage CP-54 (3 x 10^9 PFU) was spread on the transduction plates to lyse noncapsulated cells and to allow the selection of capsulated transductants. Incubation in CO_2 was continued for 36 to 48 h.

Transduction with TP-21. For the transduction of chromosomal markers, 0.1 ml of phage lysate was spread together with 0.1 ml of recipient cells onto Minimal 4NH. Recipient cells were grown from a 10% transfer of a 16-h L broth culture and incubated for 4-6 h at 30°C. Plates were incubated at 37°C and the transductants were scored after 48 h. For the transduction of Tn917-encoded MLS resistance, transducing lysates and recipient cells were mixed together and an inducing concentration of erythromycin (0.1 µg/ml) was added. The transducing mixture was then incubated for 45 min with shaking to allow the phage to adsorb. After this period of incubation 0.2 ml of the transducing mixture was added to 2.5 ml of soft NBY or L agar also containing an inducing concentration of erythromycin (0.1 µg/ml) and this was poured on either an L agar or NBY agar plate containing selective levels of antibiotic (erythromycin 2 µg/ml and lincomycin 25 µg/ml). Transductants could be scored after 16 h with B. cereus and after 24 h with B. anthracis.

Procedures used in mating experiments:

(1) Matings in broth: Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared

by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml. If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10 µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

(2) Matings on membranes: Donor and recipient cells were grown in 250-ml flasks containing 25 ml of BHI broth and incubated at 30°C on a reciprocal shaker, 80 excursions per min. Transfers (5%, v/v) from 14- to 16-h cultures were grown for 5 h. One ml of donor cells and 1 ml of recipient cells were mixed and 0.1-0.2-ml samples were spread onto Millipore DA or HA membranes (Millipore Corp., Bedford, MA) which were placed on nonselective medium for 5 hr. BHI agar was usually used if the recipients were B. anthracis, B. cereus, or B. thuringiensis. PA agar was usually used for B. subtilis, and LG agar was used when the matings involved B. subtilis natto. To determine the number of donor and recipient cells per membrane, the mixture was diluted in peptone and plated on the appropriate selective media. Control mixtures contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Plates were incubated at 30°C for 5 h to allow mating and phenotypic expression. Membranes were

subsequently transferred to agar plates containing tetracycline (for pBC16 transfer) and either rifampicin or streptomycin to select for recipients which had acquired the antibiotic resistance plasmid from the donor. To select for transfer of Tn*917*-containing plasmids, membranes were transferred to agar containing erythromycin and lincomycin, and either rifampicin or streptomycin. Colonies were scored after 1 to 2 days of incubation and transcipients were purified on the selective medium. The use of auxotrophically-marked strains facilitated unambiguous identification of transcipients. Frequency is expressed as the number of transcipients per donor.

Screening colonies for protective antigen production. Colonies were picked to plates of CA-agarose medium and incubated at 37°C in 20% CO_2 for about 16 h. A zone of precipitate formed around colonies that produced the protective antigen component of anthrax toxin (12).

Electroelution of restriction fragments from agarose gels. Following electrophoresis of the restriction endonuclease digests, the sections of the agarose gel containing DNA fragments to be eluted were cut out and then soaked in Tris-borate buffer (0.089 M Tris-hydroxide, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3) for 5 to 10 min. Electroelution of DNA was carried out using an Elutrap electro-separation chamber according to the manufacturer's instructions (Schleicher and Schuell, Inc., Keene, NH).

Southern blotting and hybridization. DNA was purified by isopycnic centrifugation in cesium chloride gradients. The DNA was radiolabelled by nick translation (22) using [α-^{32}P]-dGTP purchased from Amersham, Arlington Heights, IL, and a kit obtained from Bethesda Research Laboratories. Plasmid DNA restriction fragments separated on a 0.6% agarose gel were transferred to GeneScreen Plus nylon membranes (New England Nuclear Co.) by a modification (21) of the Southern blotting technique (25). The DNA-DNA hybridization protocol was that recommended by the supplier.

The DNA probes used for hybridization experiments were prepared according to the following procedures. pHT44 DNA (pUC18::Tn*4430*) was extracted from *E. coli* by the alkaline lysis method of Birnboim and Doly (3) as described by Maniatis et al. (18) and purified by isopycnic centrifugation in CsCl. The plasmid DNA was cut with *Kpn*I, and the 4.2-kb restriction fragment (Tn*4430*) was electroeluted as described above. The Tn*917*-containing plasmid pTV1 (31) was extracted from *B. subtilis* according to the procedure of Koehler and Thorne (10) and purified by isopycnic centrifugation in CsCl. The three *Ava*I

restriction fragments (2.2, 1.8, and 1.2 kb) representing Tn917 DNA were electroeluted from agarose gel slices as described above.

Screening colonies for fertility. A replica plate mating technique was employed to screen large numbers of transcipients for fertility. Colonies of transcipients to be tested were picked to BHI agar to form master plates. These were incubated 16 to 18 h at 30°C and the colonies were replica plated to BHI agar plates that had been spread with 0.1 ml of spores (approx 1 x 10^8 CFU) of a recipient strain. The Str^r strains, B. anthracis UM44-2 and B. cereus UM20-1, and the Rif^r strain, B. anthracis UM23-4, were used as recipients. The plates were incubated 16 to 18 h at 30°C and the mixed growth was then replica plated to agar plates containing tetracycline and the appropriate antibiotic to select for the recipient strain. Incubation at 30°C was continued. After 16 to 20 h, patches of transcipient growth were present in areas corresponding to particular colonies of transcipients on the master plate which were fertile.

Plasmid curing procedures. Strains were cured of pBC16 by growing colonies on L agar at 42°C for 24 hours. Colonies were subsequently incubated at 30°C until sporulation occurred. Individual colonies were streaked for single colony isolation and these were screened for tetracycline sensitivity. Loss of pBC16 was confirmed by plasmid analysis. Strains were cured of pXO12 by cultivation in the presence of novobiocin (6) or by serial passage at 42°C. The latter procedure was also used to cure strains of pXO1 (6, 27).

The B. anthracis strains UM44-5 harboring the deletion derivative pXO12-4 and A UM18-7 harboring the deletion derivative pXO12-3 were obtained by serial passage of UM44-4 and A UM18-6 at 42°C (19, 27). Tetracycline-sensitive colonies unable to produce toxin were confirmed by plasmid analysis to have lost pXO1 and pBC16.

Procedure for transposition of Tn917 from pTV1 in B. subtilis. A loop of growth was inoculated in a 250-ml Erlenmeyer flask containing 25 ml of LG broth and the culture was allowed to grow overnight at 37°C with shaking (100-160 rpm). A 1% transfer was then made to fresh medium and the culture was grown to log phase at 37°C. The cells were diluted 1:50 in LG broth containing erythromycin (0.1 µg/ml) and grown for a period of 4 hours in order to induce transposition of Tn917. The culture was then incubated at 45°C for 4 hours to cure the cells of pTV1, after which it was streaked for single colonies on L agar containing selective levels of erythromycin and lincomycin.

From the streak plates colonies were picked to L agar plates containing chloramphenicol to identify ones that were cured of pTV1.

Generation of deletion derivatives of pLS20::Tn917. Strains harboring a Tn917-marked pLS20 were grown in either 25 ml of BHI broth containing 1 µg of erythromycin per ml (if the plasmid was present in a B. anthracis host) or LG broth containing 1 µg of erythromycin per ml (if the plasmid was present in a B. subtilis host) at 37°C. After 8-12 hours 10% transfers of the cultures made to the same medium and this was repeated several times.

Generation of pXO12::Tn917 derivatives. To construct pXO12::Tn917 derivatives, a Tn917-carrying derivative of the B. subtilis (natto) conjugative plasmid pLS20 designated pXO503 (10) was used as a transposition vector. A mating was performed between Weybridge UM44-4(pXO1, pXO12, pBC16) and Weybridge A UM23-5(pXO503) and Tc^r MLS^r Rif^r transcipients were selected. A transcipient designated Weybridge A UM23-16 was isolated which contained pXO12, pXO503 and pBC16.

To induce transposition of Tn917 from pXO503 onto pXO12, Weybridge A UM23-16(pXO12, pXO503, pBC16) was grown in the presence of inducing concentrations of erythromycin (20 ng/ml) and mitomycin C (10 ng/ml) (30). Cells from the induced culture were used to prepare broth mating mixtures with B. cereus 569 UM20-1 as recipient. Previous work in our laboratory has shown that, in contrast to pXO12, pLS20 mediates transfer of itself and other plasmids more readily on solid medium than in broth (T. M. Koehler, Ph.D. thesis, University of Massachusetts, Amherst, 1987). Therefore, these matings would favor the transfer of pXO12 over the pLS20 derivative pXO503. Representative MLS^r Str^r transcipients from the above mating were streaked for single colony isolation and then examined for plasmid content to identify isolates which contained pXO12::Tn917 derivatives.

Generation of pXO2::Tn917-containing strains. To construct a pXO2::Tn917 derivative a mating was performed between B. cereus 569 UM20-13(pXO12-2) and B. anthracis 6602(pXO2), and prototrophic MLS^r transcipients were selected on Min IC agar containing erythromycin. From this mating an MLS^r Cap^+ transcipient designated 6602 UM5 was isolated which contained the plasmids pXO2 and pXO12-2. To effect the transposition of Tn917 from pXO12-2 to pXO2, a mating was carried out between 6602 UM5(pXO2, pXO12-2) and Weybridge UM44-2. MLS^r Str^r transcipients were selected and these were screened for capsule production. An MLS^r Str^r Cap^+ transcipient designated

Weybridge UM44-10 was isolated which harbored only pXO2-8, a pXO2::Tn*917* derivative.

To allow us to determine the frequency of pXO12-mediated transfer of the pXO2::Tn*917* derivative, we introduced pXO12 into Weybridge UM44-10(pXO2-8) by mating it with the donor, Weybridge A UM23-8(pXO12, pBC16). A transcipient, designated Weybridge UM44-11, was isolated which contained pXO2-8, pXO12, and pBC16.

RESULTS AND DISCUSSION

I. Involvement of Tn4430 in the transfer of Bacillus anthracis plasmids mediated by the Bacillus thuringiensis plasmid pXO12

Recent reports from our laboratory have concerned the identification and characterization of six self-transmissible plasmids from five different subspecies of *Bacillus thuringiensis* (1, 21). One of these plasmids, designated pXO12 (112.5 kb), was isolated from strain 4042A of *B. thuringiensis* subspecies *thuringiensis*. Plasmid pXO12 is capable of mediating its own transfer as well as the transfer of a large range of *Bacillus* plasmids among strains of *Bacillus anthracis*, *Bacillus cereus*, and *B. thuringiensis*. In addition to conjugal transfer functions, pXO12 also encodes production of the insecticidal toxin known as the delta-endotoxin or the parasporal crystal (Cry^+). Because the *B. thuringiensis* conjugative plasmids studied in our laboratory have no known selectable markers, it has been necessary to assess indirectly the conjugal activities they confer upon their hosts, i.e., by selecting for the transfer of the *B. cereus* tetracycline resistance plasmid pBC16 (2) and then examining Tc^r transcipients for acquisition of additional plasmids. In this report we describe the construction of pXO12 derivatives that harbor the *Streptococcus faecalis* erythromycin resistance transposon Tn*917* (20, 29). The isolation of marked derivatives of pXO12 has enabled us to determine the transfer frequency of this plasmid directly during mating. Mating experiments have shown that the pXO12::Tn*917* derivatives transfer at frequencies similar to the frequency of pXO12-mediated transfer of pBC16.

The widespread occurrence of large self-transmissible plasmids among *B. thuringiensis* strains suggests that conjugation may be an important means of

plasmid dissemination in naturally occurring Bacillus populations (4). In the laboratory, this mating system has provided us with an efficient method of shuttling a wide range of plasmids among B. thuringiensis, B. cereus, and B. anthracis. For example, the utility of this conjugation system in the genetic analysis of the B. anthracis toxin and capsule plasmids, pXO1 and pXO2, respectively, has been well documented (1, 6, 21, 27).

Prior to the development of this mating system, the most reliable genetic exchange system available to these three Bacillus species was transduction (23). Therefore, the importance of this system as an additional means of genetic exchange among these organisms warrants further genetic and physical analysis of these conjugative plasmids. We have been interested in determining the mechanism of pXO12-mediated transfer of both large and small plasmids. To this end, we have monitored the transfer of pBC16 (4.2 kb) and the B. anthracis toxin and capsule plasmids, pXO1 (168 kb) and pXO2 (85.6 kb), respectively.

Physical analysis of the transferred plasmids suggested that pBC16 was transferred by the process of donation while the large B. anthracis plasmids were transferred by the process of conduction. The transfer of pXO1 and pXO2 involved transposition of the B. thuringiensis transposon Tn4430 (14, 17) from pXO12 onto these plasmids. DNA-DNA hybridization experiments demonstrated that Tn4430 was located on a 16.0-kb AvaI fragment of pXO12. Examination of Tra^- and Cry^- derivatives of pXO12 showed that this fragment also harbored information involved in crystal formation and was close to DNA sequences involved in conjugal transfer ability. Thus, it appears that the aforementioned genetic elements are located close together on pXO12.

Transfer of the B. anthracis resident plasmids by pXO12. We previously reported transfer of the tetracycline resistance plasmid pBC16 and the B. anthracis plasmids pXO1 and pXO2 from strains harboring the B. thuringiensis fertility plasmid pXO12 (1, 6, 27). More recent experiments suggest that the frequency of transfer of the small plasmid pBC16 is considerably higher than the frequency of transfer of the large plasmids pXO1 and pXO2. As shown in Table 2A the number of Tc^r transcipients ranged from 10^3 to 10^5 per ml, whereas the number of Tc^r transcipients that simultaneously inherited pXO1 was only 0.3% to 0.4% of the total. Similar results were obtained in pXO12-mediated transfer of pXO2 (6).

To compare more accurately the frequency of pXO2 transfer with that of pBC16, Weybridge UM44-11, which contained pXO2-8, pXO12, and pBC16, was mated with Weybridge A UM23-4. This allowed direct selection of transcipients which acquired pXO2-8 or pBC16 by plating on agar containing rifampicin and either erythromycin and lincomycin or tetracycline, respectively. The results of two independent matings are shown in Table 2B. The number of Tc^r transcipients obtained was approximately 2×10^5 per ml, and the number of MLS^r transcipients ranged from 4.0×10^2 to 2.7×10^3. Interestingly, only 45% to 46% of the MLS^r transcipients were Cap^+. Thus, the number of transcipients that inherited pXO2-8 was 200- to 1000-fold fewer than the number that inherited pBC16. Examination of the plasmid profile of representative MLS^r Cap^- transcipients showed that they contained a pXO12 plasmid which carried a copy of Tn917. Transcipients which were MLS^r Cap^+ were found to have inherited pXO12 and pXO2::Tn917 plasmids which comigrated with the respective plasmids of the donor strain.

Plasmids pXO1 and pXO2 contain Tn4430 after pXO12-mediated mobilization. Plasmid analysis was performed on representative Tox^+ or Cap^+ transcipients to confirm the acquisition of pXO1 or pXO2 in addition to pBC16. These analyses showed that there were a variety of plasmid profiles represented among the transcipients (Figs. 1A and 2A). The majority of Tox^+ or Cap^+ transcipients examined harbored pXO12 (Cry^+) and either pXO1 or pXO2 which on agarose gels appeared to be indistinguishable from the respective plasmids present in the donor strains. However, other transcipients which were Cry^+ and either Tox^+ or Cap^+ contained a single large plasmid migrating above the chromosomal DNA in agarose gels. This suggested the formation of cointegrate plasmids (Fig. 1A, lane 6; Fig. 2A lane 5). In contrast to the variability of Tox^+ and Cap^+ transcipients, all Tc^r transcipients inherited plasmid DNA which comigrated with pBC16 from the donor strains.

The high frequency of pBC16 transfer suggests that transfer of this plasmid occurs by donation. In contrast, the lower frequency of transcipients which acquired pXO1 or pXO2 suggests that transfer of these large plasmids may be by conduction; a process which requires physical contact between the conjugative and nonconjugative plasmid. Since in other conjugation systems physical contact between the fertility plasmid and other mobilizable plasmids is often mediated by transposable elements (5), we examined pXO1 and pXO2 plasmids before and after mobilization for hybridization to Tn4430. Plasmid

DNA from the agarose gels in Figures 1A and 2A was transferred to nylon membranes and probed with ^{32}P-labelled Tn*4430*. The results of these hybridizations are shown in Figures 1B and 2B. Tn*4430* hybridized to pXO1 from 6 out of 7 Tox$^+$ transcipients tested and to pXO2 from 7 out of 8 Cap$^+$ transcipients tested. There was no detectable hybridization with pXO1 or pXO2 before mobilization (Fig. 1B, lanes 1 and 8; Fig. 2B, lanes 1, 6, and 10). As expected, Tn*4430* also hybridized to the presumed cointegrate plasmids (Fig. 1B, lane 6; Fig. 2B, lane 5).

To determine the sites of insertion of Tn*4430*, we examined several pXO1 and pXO2 plasmids by restriction endonuclease digestion. To facilitate isolation of pXO1 from various Tc^r transcipients generated from matings described in Table 2, we cured them of pXO12 and pBC16 sequentially by cultivation in the presence of novobiocin followed by serial passage at 42°C. In this way strains were obtained which harbored pXO1 alone. pXO1 DNA was isolated and digested with *Eco*RI or *Kpn*I and electrophoresed in 0.7% agarose gels. The restriction patterns of the pXO1 derivatives were compared to those of similarly digested pXO1 from Weybridge A UM2 which was taken as wild type. As shown in Figure 3A there were noticeable differences in the restriction patterns of wild-type pXO1 and the toxin plasmid from the transcipients. For example, the 7.95-kb *Eco*RI fragment present in wild-type pXO1 was missing from pXO1-1 of the donor strain A UM2-3, and two new fragments, 4.15 kb and 6.45 kb, were present (Fig. 3A, lanes 7 and 8). There were further alterations in pXO1-1 after being mobilized by pXO12. For example, in pXO1-2 from UM44-6, the 6.45-kb *Eco*RI fragment was replaced with a 10.6-kb fragment (Fig. 3A, lane 9). In pXO1-3 from A UM23-9, the 5.6-kb *Eco*RI fragment was replaced with a 9.8-kb fragment (Fig. 3A, lane 10). Comparison of *Kpn*I digests of pXO1, pXO1-1, pXO1-2, and pXO1-3 showed that the latter two plasmids had acquired a 4.2-kb fragment which comigrated with the *Kpn*I fragment that we have shown to be Tn*4330* (Fig. 3A, lanes 5 and 6). When the DNA fragments from the gel in Figure 3A were transferred to nylon membranes and probed with ^{32}P-labelled Tn*4430*, only those fragments which were unique to pXO1-2 and pXO1-3 showed homology to the transposon (Fig. 3B).

To determine the sites of insertion of Tn*4430* in pXO2 after mobilization, we examined pXO2 DNA before and after transfer by pXO12. To facilitate isolation of pXO2 DNA from transcipients which also carried pXO12 and pBC16, we transferred pXO2 by transduction with phage CP-51 to *B. anthracis* which had

been cured of all plasmids. Plasmid DNA from representative Cap^+ transductants was isolated and digested with restriction endonucleases KpnI or EcoRI. Figure 4A compares KpnI and EcoR1 digestion patterns of the pXO2 plasmids, designated pXO2-1 and pXO2-2, originally found in the Cap^+ transcipients, B. cereus 569 UM20-6 and 569 UM20-2, respectively, to similarly digested pXO2 from B. anthracis 6602. Digestion of pXO2-1 and pXO2-2 with KpnI showed that these plasmids had acquired a 4.2-kb fragment which comigrated with the 4.2-kb KpnI fragment of pXO12 (Fig.4A, lanes 4 and 5). EcoR1-digested pXO2-1 showed a loss of the 6.1-kb fragment present in digests of wild-type pXO2 and the presence of a new 10.3-kb fragment (Fig. 4A, lane 7). Examination of EcoR1-digested pXO2-2 showed that it had lost the 6.1-kb and 9.4-kb fragments and gained two new fragments of 5.2 kb and 10.3 kb (Fig. 4A, lane 8). The pXO2 DNA fragments of Fig. 4A were transferred to nylon membranes and probed with ^{32}P labelled Tn4430. The fragments that showed homology to Tn4430 were unique to pXO2-1 and pXO2-2 (Fig. 4B).

Plasmid pBC16 is unaltered after transfer. As mentioned earlier the high frequency of transfer of pBC16 suggests that this plasmid is transferred by donation. Since transfer of nonconjugative plasmids by donation does not require physical contact with a fertility plasmid, there should not be any alteration in pBC16 after it has been transferred. To confirm this we examined pBC16 before and after mobilization by pXO12. pBC16 from each of two independently derived B. anthracis transcipients was moved into plasmid-free Weybridge A UM23-3 by transduction with CP-51, and plasmid DNA was isolated from transductants. As a control we used pBC16 which had been transduced into B. anthracis but which had not been exposed to pXO12 in our laboratory. Restriction analysis of the various pBC16 plasmids confirmed that there were no alterations after transfer, and tests for hybridization with ^{32}P-labelled pXO12 showed that there was no homology between the two plasmids (B. D. Green, Ph.D. thesis, University of Massachusetts, Amherst, 1988).

Generation of cointegrate plasmids of pXO12 and the B. anthracis resident plasmids. From mating mixtures that were screened for cotransfer of the Tox^+ and Cry^+ phenotypes from donors carrying pXO1 and pXO12, some of the transcipients that inherited both phenotypes did not contain pXO1 or pXO12. Instead they contained a plasmid which was higher in molecular weight than either of the two expected plasmids. This suggested that in the course of mating cointegrate plasmids were formed between pXO1 and pXO12. To test the

ability of these strains to transfer the large plasmid and pBC16, matings were performed using two independent B. anthracis transcipients, Weybridge UM44-9 and Weybridge A UM23-12, as donors to cured strains of B. anthracis. The results of these matings are shown in Table 3A. The donor strains harboring the putative pXO1::pXO12 cointegrates transferred pBC16 at frequencies comparable to those of pXO12-mediated transfer of pBC16 (10^{-2} - 10^{-4}). However, the proportion of Tc^r transcipients which were also Tox^+, i.e., approximately 50%, was much larger than had been observed previously. In addition, all of the Tox^+ transcipients were also Cry^+. Plasmid analysis revealed that the Tox^+ Cry^+ transcipients inherited a large plasmid that comigrated in electrophoretic gels with the suspected cointegrate present in the donors. None of several Tox^- Cry^- transcipients examined carried a similar large plasmid.

Results analogous to those with pXO1 were also obtained with pXO2. A Cap^+ Cry^+ transcipient designated B. cereus 569 UM20-4 was obtained which harbored pBC16 and an apparent cointegrate of pXO2 and pXO12 (Fig. 2A lane 5). When this strain was used as the donor in matings with Weybridge A UM23-4, pBC16 was transferred at a frequency comparable to that of donor strains containing wild-type pXO12 (Table 3B). The proportion of Tc^r transcipients which simultaneously acquired the ability to make capsules was much larger than had been observed previously with donor strains that contained wild-type pXO2 and pXO12. Plasmid analysis of Tc^r Cap^+ Cry^+ transcipients revealed that these strains had inherited, in addition to pBC16, a single large plasmid which migrated at the same rate as the presumed cointegrate plasmid in the donor strain. The large plasmid was not found in several Tc^r Cap^- Cry^- transcipients examined.

Localization of Tn4430 on pXO12. The above results suggest that Tn4430 has a role in pXO12-mediated transfer of the large B. anthracis plasmids. Experiments have shown that there is a copy of Tn4430 on pXO12 as well as three other self-transmissible B. thuringiensis plasmids that have been investigated in our laboratory (21). Therefore, it would be interesting to determine whether Tn4430 is near DNA sequences carrying information required for conjugal transfer ability. To determine the location of Tn4430 on pXO12, we probed a blot of the AvaI restriction digest of pXO12 for hybridization with ^{32}P-labelled Tn4430. This experiment showed that there was strong homology between Tn4430 and the 16.0-kb AvaI fragment of pXO12 (L. A. Battisti, Ph.D. thesis, University of Massachusetts, Amherst, 1988). As discussed

below, this fragment was found to be adjacent to sequences commonly lost in Tra^- deletion derivatives of pXO12.

Restriction analysis of transfer deficient deletion derivatives of pXO12. We isolated two Tra^- Cry^+ deletion derivatives of pXO12, designated pXO12-3 and pXO12-4, following growth of pXO12-containing B. anthracis strains at 42°C. Restriction analysis of the two derivatives revealed that both had lost the 48.5-kb AvaI restriction fragment (Fig. 5, lanes 3 and 4). These results suggested that the 48.5-kb AvaI fragment of pXO12 carries information required for conjugal transfer ability. The simultaneous loss of the 48.5-kb and the 16.0-kb AvaI fragments to produce pXO12-4 (Fig. 5, lane 4) suggests that these fragments are adjacent on pXO12.

Physical and genetic analysis of pXO12::Tn917 derivatives. Two Tn917-carrying derivatives of pXO12, designated pXO12-1 and pXO12-2, were isolated from the MLS^r Str^r transcipients, Weybridge A UM23-18 and B. cereus 569 UM20-13, respectively. Restriction analysis with AvaI confirmed the insertion of Tn917 DNA into the two plasmids. Tn917 was found to contain four AvaI cleavage sites when propagated in B. subtilis PSL1(pTV1) and in B. anthracis Weybridge A UM23-5(pXO503). As reported earlier (20), two of the four AvaI sites in Tn917 are symmetrically located five bp from each end of its terminal inverted repeats. Thus, digestion of Tn917 with AvaI produced the three distinct fragments approximately 2.2, 1.8, and 1.2 kb in size (Fig. 6A, lanes 1 and 2). Perkins and Youngman (20) reported a similar cleavage pattern for AvaI-digested Tn917 DNA that had been subcloned in an E. coli host. Digestion of pXO12-1 and pXO12-2 with AvaI generated three new restriction fragments that comigrated with the 2.2-, 1.8-, and 1.2-kb fragments of Tn917 (Fig. 6A, lanes 4 and 5). DNA-DNA hybridization experiments corroborated these results; ^{32}P-labelled Tn917 DNA isolated from pTV1 hybridized to the 2.2-, 1.8- and 1.2-kb fragments of the pXO12::Tn917 derivatives (Fig. 6B, lanes 4 and 5).

Mating experiments showed that B. anthracis Weybridge A UM23-18 and B. cereus 569 UM20-13 could both act as donors of the pXO12::Tn917 derivatives (Table 4). In addition, phase microscopy revealed that both donors were Cry^-. Restriction analysis of pXO12-1 and pXO12-2 showed that the Tra^+ Cry^- plasmids had both lost the 16.0-kb AvaI fragment of pXO12 (Fig. 6A, lanes 4 and 5), suggesting that this fragment carries information required for the synthesis of parasporal crystals. Absence of the 16.0-kb AvaI fragment from pXO12-2 suggests that insertion of Tn917 occurred in that fragment and that the inser-

tion generated two new AvaI fragments of approximately 8.3 and 4.2 kb (Fig. 6A, lane 5). Because of the number of DNA alterations exhibited by pXO12-1 (Fig. 6A, lane 4), the site of Tn*917* insertion in that plasmid could not be determined by this analysis.

Transfer frequency of pXO12::Tn*917* derivatives. The Tn*917*-carrying derivatives of pXO12 were used to determine the transfer frequency of pXO12 by monitoring the transfer of MLS resistance. Matings were performed using Weybridge A UM23-18(pXO12-1) and *B. cereus* 569 UM20-13(pXO12-2) as donors of the pXO12::Tn*917* derivatives to erythromycin-sensitive *B. anthracis* and *B. cereus* recipients. Similar matings in which the donors contained wild-type pXO12 and pBC16 were included in the experiment to allow comparison of the frequencies of pXO12::Tn*917* transfer with the frequencies of pXO12-mediated transfer of pBC16. The results are shown in Table 4. The transfer frequencies of the pXO12::Tn*917* derivatives were comparable to the frequencies of pXO12-mediated transfer of pBC16.

The results presented here suggest that pXO12-mediated transfer of the high molecular-weight *B. anthracis* plasmids pXO1 and pXO2 occurs by conduction. The evidence for this is two-fold: (i) the transfer frequency of pXO1 or pXO2 was very low relative to the transfer frequency of pXO12::Tn*917* or pBC16 and (ii) pXO1 and pXO2 in a majority of transcipients which acquired either plasmid contained a copy of Tn*4430*. The frequencies of pXO12-mediated transfer of pBC16 ranged from 1.6×10^{-4} to 7.1×10^{-2}, but only 0.3% to 0.4% of the Tc^r transcipients acquired pXO1 or pXO2 in addition to pBC16. The implied lower frequency of pXO12-mediated transfer of the *B. anthracis* plasmids compared to that of pBC16 was confirmed by matings using a donor strain which contained pXO12, pBC16, and a pXO2 plasmid which had been tagged with Tn*917*. The results of such matings showed that the number of transcipients which inherited the pXO2 derivative was 200- to 1000-fold lower than the number which inherited pBC16.

Transcipients that had acquired pXO1 or pXO2 by mating were examined for the presence of Tn*4430* on the transferred plasmids. In 6 out of 7 $pXO1^+$ transcipients and in 7 out of 8 $pXO2^+$ transcipients that were examined the transferred plasmids contained a copy of the transposon.

Several transcipients obtained from independent matings contained presumed cointegrate plasmids of pXO12 and the *B. anthracis* plasmid pXO1 or

pXO2. When strains containing these plasmids were used as donors in matings with cured strains of B. anthracis, the proportion of Tc^r transcipients which were also Tox^+ was higher than normal. For example, Weybridge UM44-9(pXO1::pXO12, pBC16) and Weybridge A UM23-12(pXO1::pXO12, pBC16) transferred pBC16 at frequencies between 10^{-2} and 10^{-4}. Approximately 50% of the Tc^r transcipients tested were also Tox^+. All of the Tox^+ transcipients examined were also Cry^+ and harbored a plasmid indistinguishable from that found in the donor strain. Similarly, B. cereus 569 UM20-4, a strain carrying a putative pXO2::pXO12 cointegrate, transferred pBC16 at a frequency of 1.7×10^{-4}, and approximately 20% of the Tc^r transcipients were Cap^+. All of the Cap^+ transcipients were Cry^+ and harbored a plasmid which was indistinguishable from the large plasmid in the donor strain.

These studies suggest that the function of Tn4430 in the mobilization of pXO1 and pXO2 is to mediate the formation of cointegrate molecules between the fertility plasmid pXO12 and the nonconjugative B. anthracis plasmids. The cointegrate plasmid is then transferred to recipient cells where it usually resolves into pXO12 and the respective B. anthracis plasmid. However, our results have shown that in some instances these cointegrate plasmids are stably maintained in transcipient cells. We have also found that Tn4430 is not unique in its function of mediating the formation of cointegrate plasmids with pXO12. In experiments not described here, we used a pXO12::Tn917 plasmid to mobilize pXO2. Several Cap^+ transcipients obtained from these matings inherited a pXO2 plasmid in which a copy of Tn917, but not Tn4430, was inserted.

The results presented here demonstrate that Tn4430 is located in the same vicinity on pXO12 as the crystal gene and information involved in conjugal transfer ability. Lereclus et al. (16) reported a model for the structural organization of Tn4430 (4.2 kb), a crystal gene (ca. 3.7 kb) and two sets of inverted repeats, IR1 (1.7 kb) and IR2 (2.1 kb), on plasmid pBT42, indigenous to the berliner strain of B. thuringiensis. They found that these genetic elements were arranged to form a composite transposon-like element on the plasmid. Tn4430 and the crystal gene were separated by one copy of IR1, and these three elements were flanked on each side by another copy of IR1 (16). Similarly, Kronstad and Whiteley (17) have found that two sets of inverted repeat sequences, IR2150 and IR1750, flank the crystal toxin gene on the 75-kb plasmid of B. thuringiensis subsp. kurstaki HD73. Because of the

molecular relatedness of crystal-encoding plasmids in general (4, 13), it is likely that the crystal gene and Tn4430 are similarly arranged on pXO12. Further studies are needed to (i) map the exact location of the crystal gene on pXO12 and (ii) to determine whether there are also copies of inverted repeat elements present on this region of pXO12.

Studies by Lereclus et. al. (16, 17) have shown that Tn4430 promotes deletions in DNA sequences adjacent to its endpoints. It is possible then that Tn4430 or insertion elements related to IR1 are responsible for the spontaneous loss of DNA sequences on this region of pXO12. Therefore, Tn4430 may be directly responsible for generating deletion derivatives of pXO12 that are Tra$^-$.

II. Transformation of B. cereus with plasmid DNA

In recent years a number of reports have appeared in the literature on methods for transforming B. anthracis, B. thuringiensis, or B. cereus. We have tried unsuccessfully to transform B. cereus and B. anthracis by most, if not all, of the methods that have been reported. This has been the experience with most laboratories that have tried to use the transformation methods that have been reported. In fact, in some instances, the authors of the papers describing the methods have been unable to repeat their results. The main problem in transforming these organisms appears to be in the process of regenerating protoplasts.

In my last annual report (Annual Progress Report, July 1987) I reported that we had some success in adapting the procedure of Heierson, et al. (7) to B. cereus. However, the yields of transformants obtained with the tetracycline resistance plasmid pBC16 were very low, and we were unsuccessful in obtaining any transformants with larger plasmids such as pTV1.

During the past year we have had greater success with an unpublished procedure for transformation of B. thuringiensis protoplasts with plasmid DNA (Dietmar Schall, personal communication). We were successful in adapting the procedure to B. cereus 569 and were able to transform protoplasts of that organism with pBC16 and pTV1 DNA. Although we were not successful in reproducibly transforming B. anthracis by this procedure, once we had transformed B. cereus with a plasmid such as pTV1 we could then transfer it to B. anthracis by CP-51-mediated transduction.

The procedure of Schall, as we adapted it for B. cereus, is given below.

Reagents for transformation of Bacillus cereus:

A. HGP-broth (without trace elements):

	g/liter
Tryptone	10
Yeast extract	5
NaCl	5
Dextrose	5
Citric acid.H_2O	0.11

Adjust pH to 7.0 with 10 N NaOH and bring to desired volume with H_2O. Autoclave 15-20 min.

2X Trace elements (without CuSO4):

	g/100 ml
$CaCl_2.2H_2O$	1.6
$ZnCl_2$	0.1
$MnCl_2.4H_2O$	1.0
$FeCl_3.6H_2O$	0.1

Filter before use. May have to add 6 N HCl to keep Fe^{+++} in solution.

2X $CuSO_4$: Dissolve 0.25 g/100 ml in distilled H_2O and filter before use.

Complete HGP-broth: Add 5 ml of 2X trace elements and 5 ml of 2X $CuSO_4$ per liter of HGP-broth.

B. AP broth:

	g/liter
Tryptone	10
Yeast extract	5
NaCl	5
Dextrose	1

Adjust pH to 7.4 with 10 N NaOH and bring to desired volume with distilled H_2O. Autoclave 15-20 min.

C. Buffers for protoplast formation and dilutions:

1. STM:

	g/100 ml	Concentration
Sucrose	17.1	0.5 M
Tris-OH	0.36	30 mM
$MgCl_2.H_2O$	0.10	5 mM

Adjust pH to 8.0 with 6 N HCl, bring to volume with distilled H_2O, and store at 4°C. Filter before use.

2. STML: STM with 0.5 mg of lysozyme per ml. Filter sterilize before using.

3. 2X SMM:

	g/100 ml	Concentration
Sucrose	34.2	1 M
Maleic acid	0.46	40 mM
$MgCl_2.6H_2O$	0.81	40 mM

Adjust pH to 6.5 with 10 N NaOH, bring to volume with distilled H_2O, filter sterilize, and store at 4°C

4. 4X Antibiotic Media No. 3: Add 70 g Difco Antibiotic Medium No. 3 to 1 liter H_2O. Autoclave 15-20 min.

5. SMMP: Mix equal volumes of filtered 2X SMM and sterile 4X Antibiotic Medium No. 3.

6. SMMP M/L: SMMP with 2500 units of mutanolysin and 1 mg of lysozyme per ml. Filter before use. (Determine optimum amount of each lot of mutanolysin; lots vary in activity.

7. 40% PEG 8000: Add 4 g PEG 8000 to 6 ml filtered 1X SMM. Steam for 15-20 min to dissolve.

D. Regeneration medium for Bacillus cereus:

1. 2X regeneration agar:

	g/300 ml
Gelatin	15
Soluble starch (Difco)	9
Difco Agar	15

Mix dry ingredients. Add 150 ml H_2O and quickly mix by vigorous stirring. Add another 140 ml H_2O and mix. Autoclave 25-30 min.

2. 4X Sucrose: Add 612 g sucrose to 450 ml H_2O. Dissolve completely and adjust volume to 900 ml with

H_2O. Dispense into 150 ml-portions and autoclave for 15 min.

3. 4X DS nutrient broth for regeneration:

	g/900 ml
Tryptone	36
Yeast extract	18
Dextrose	18
NaCl	7.2
Sodium citrate.$2H_2O$	8.22
$MgCl_2.6H_2O$	1.8
$CaCl_2.2H_2O$	1.44

Adjust pH to 7.0 with 10 N NaOH, bring to desired volume (steam if necessary), and dispense 150-ml portions. Autoclave 15 min.

4. Regeneration medium:

Add 150 ml 4X sucrose and 150 ml 4X DS nutrient broth to 300 ml 2X regeneration agar. Keep at 55°C until antibiotics are added. Pour immediately after addition of antibiotics.

RT15C1: Regeneration medium with 15 µg of tetracycline and 1 µg of chloramphenicol per ml.

RC15: Regeneration medium with 15 µg of chloramphenicol per ml.

Preparation of B. cereus cultures for protoplast formation:

Overnight culture: Inoculate 100 ml complete HGP-broth in a 250-ml Erlenmeyer flask with a loop of spores. Incubate on a shaker (150 rpm) at 30°C for 16 hr.

Autolysis culture: Transfer 1 ml of an overnight culture to 100 ml of AP-broth in a 500-ml Erlenmeyer flask. Incubate on a shaker (300 rpm) at 30°C for 4-5 hr. Test pH to determine optimal growth stage (We obtained good protoplasts with Bacillus cereus 569 UM20-1 at pH 6.2-6.5 after culture had been initially below pH 6.0).

Bacillus cereus protoplast formation:

Autolysis method: Centrifuge 5 ml autolysis culture in sterile 16 x 100-mm screw-cap tube (15 min, clinical centrifuge), resuspend cells in 1 ml STML, and incubate 1 hr at 30°C statically. Then add 1 ml SMMP and incubate an additional 30 min. at 30°C statically.

Mutanolysin/lysozyme method: Centrifuge 5 ml overnight culture (15 min, clinical centrifuge), resuspend cells in 1 ml SMMP M/L, incubate 2 hrs at 30°C (should observe 75 to 99% protoplasts).

Transformation:

1. Wash protoplasts 2 times with 5 ml of SMMP. Each time pellet the protoplasts by centrifugation for 10 min at 3500 rpm. Note: Make sure to gently and completely resuspend pellet
2. Gently mix:
   0.25 ml protoplast suspension
   10-100 µl plasmid extract
   0.75 ml 40% PEG 8000 in 1X SMM
   Mix by rolling tube gently.
3. Incubate 5 min at room temperature.
4. Gently add 5 ml SMMP and centrifuge at room temperature for 10-15 min at 2500 rpm,
5. Resuspend pellet in 1 ml SMMP and incubate 5 h at 30°C statically to allow phenotypic expression.
6. Spread 0.1 ml "transformed" protoplast suspension or appropriate dilutions prepared in SMMP onto RT15C1 plates (for selecting tetracycline-resistant transformants) or onto RC15 plates (for selecting chloramphenicol-resistant transformants). Incubate plates 3-5 days at 30°C. The number of transformants growing on regeneration medium appeared to increase when older plates (stored at room temperature for one or two weeks) were used.

Results of transforming B. cereus 569 UM20-1 with pBC16 DNA:

Examples of data from tranformation experiments in which B. cereus was transformed with pBC16 DNA are given below:

Method	µg of DNA/ml	Tranfsformants/ml
Mutanolysin/lysozyme method:	0.5	3.2×10^3
	1.0	4.6×10^3
	2.0	7.9×10^3
	5.0	7.3×10^3
Autolysis method:	2.0	1.1×10^3

When pTV1 DNA was used to transform B. cereus by the above procedure the efficiency was much lower. Only 10 to 40 transformants per ml were obtained.

Transformation of B. cereus and B. anthracis by electroporation:

Much better results were obtained by electroporation. The procedure was a modification of that supplied by Dr. Gary Dunny (personal communication). Some examples of data obtained in electroporation experiments are given below:

Strain	DNA	Transformants/ml
B. cereus 569 UM20-1	pBC16 (1.25 µg/ml)	5.0×10^4
B. anthracis UM23-2	pBC16 (1.25 µg/ml)	1.1×10^4
B. anthracis UM23	pBC16 (1.25 µg/ml)	7.5×10^3
B. cereus 569 UM20-1	pTV1 (2.5 µg/ml)	1.2×10^2
B. anthracis UM23-1	pTV1 (2.5 µg/ml)	3.0×10^1

The electroporation method is much easier and faster than the protoplast transformation method. It will be the method of choice in the future. With both methods the efficiency of transformation decreased dramatically as the size of the plasmid DNA increased. However, it seems likely that we will be able to modify the electroporation procedure and increase the efficiency of transformation.

III. Transposon mutagenesis in B. anthracis with the transposition selection vector pTV1

pTV1 is a transposition selection vector developed by Youngman (31). This 12.4-kb plasmid contains the Streptococcus faecalis transposon Tn917, which carries an erythromycin-inducible gene for MLS resistance. The plasmid also carries a chloramphenicol resistance determinant and is temperature sensitive for replication. It has been used to carry out transposon mutagenesis in B. subtilis and other Bacillus species into which it can be introduced by transformation or transduction. The fact that we have been able to transform B. cereus and B. anthracis by the protoplast transformation method described in this report as well as by electroporation has opened up the possbility of using pTV1 for transposon mutagenesis in B. anthracis.

Stability of pTV1 in B. anthracis. We were able to introduce pTV1 into B. anthracis directly by transformation or electroporation or indirectly by first putting it into B. cereus by transformation or electroporation and then transferring it to B. anthracis by CP-51-mediated transduction. An electrophoretic gel showing plasmid pTV1 in Weybridge A UM23 is shown in Fig. 7. In this instance pTV1 was transferred into B. anthracis by transduction from B. cereus. However, the results were the same whether the plasmid was put into B. anthracis by transduction or transformation.

Spores of transductants or transformants carrying pTV1 were grown in the presence of a selective concentration of chloramphenicol (15 µg/ml). When such spores were plated on L agar containing selective concentrations of chloramphenicol or erythromycin and lincomycin, 95 to 100 per cent were shown to be resistant to all three antibiotics, indicating that pTV1 was present. The presence of pTV1 was confirmed by plasmid analysis of cultures grown from eight randomly chosen colonies derived from the infected spores. Thus, there was no difficulty in obtaining populations of spores carrying pTV1.

Similarly, we demonstrated that pTV1 was maintained in B. anthracis cells following growth at 30°C in the absence of antibiotics. However, as shown below, the plasmid was lost from cells during growth at 43°C. When pTV1 is used for transposon mutagenesis in B. subtilis, incubation temperatures of 46° to 50°C are used to cure cells of the plasmid. However, B. anthracis does not grow above 43°C on any medium we have tested. The efficiency of curing B. anthracis cells of pTV1 at 43°C was not as high as curing efficiencies obtained with B. subtilis at the higher temperatures. Nevertheless, as shown below, the curing frequencies were sufficiently high to allow the isolation of transposants.

Selection of chromosomal insertions. Approximately 5 x 10^8 spores of B. anthracis Weybridge A UM23-2(pXO1)$^-$(pTV1) were inoculated into 25 ml of BHI-glycerol broth containing 15 µg of chloramphenicol (Cm) and 0.1 µg of erythromycin (Em) per ml (the latter to induce transposition) in a 250-ml cotton-plugged flask which was incubated at 30°C on a shaker (130 rpm). After 16 to 18 hours 0.25 ml was transferred to 25 ml of BHI-glycerol broth containing selective levels of Em (1 µg/ml) and lincomycin (Lm, 25 µg/ml) and no chloramphenicol. The transferred culture was incubated in a 43°C water bath with shaking (160 rpm) for 8 hours. The culture was transferred in a similar manner three additional times at intervals of 8 to 12 hours.

After 4 transfers to BHI-glycerol broth containing selective levels of Em and Lm at 43°C, 0.25 ml was transferred to 25 ml of L broth (no antibiotics) and incubated on a shaker at 30°C for three to 4 days to allow sporulation, which was monitored by phase microscopy. Once sporulation had occurred, the culture was centrifuged at 10,000 rpm for 10 min at 4°C (Sorvall SS-34 rotor) and the spores were resuspended in 5 ml of sterile water. The resuspended spores were heat-shocked (65°C, 30 min) and 0.1-ml samples of appropriate dilutions were spread on plates of L-agar containing 0.1 µg of Em per ml. The plates were incubated at 30°C for 24 hours.

Cells in which transposition of Tn917 and loss of pTV1 had occurred were selected by replica plating the colonies to L agar containing selective concentrations of erythromycin and lincomycin and to L agar containing 15 µg of chloramphenicol per ml. The Em^r Lm^r Cm^s colonies were screened for auxotrophic mutants on minimal XO agar, and mutant phenotypes were determined by supplementing minimal XO agar with appropriate nutrients.

When spores were prepared by the mutagenesis procedure described above, 90% or more were found to be Em^r Lm^r and Cm^s. Three out of 870 colonies tested were found to be auxotrophic mutants. One of the mutants was characterized as a purine mutant (responded very well to adenine and less well to guanine, and the other two mutants responded to riboflavin.

To determine whether the purine (Pur^-) and riboflavin (Rib^-) mutants resulted from Tn917 insertions or whether they resulted from coincidental spontaneous mutations, transductions with CP-51 were performed as follows:

(i) CP-51 was propagated on each of the mutants and used to transduce Bacillus anthracis UM23 Ura^- with selection for Em^r Lm^r transductants which were then tested for auxotrophic requirements. When phage was propagated on the Pur^- mutant, 25 out of 26 Em^r Lm^r transductants tested were shown to be Ura^- Pur^-. Similarly, when phage was propagated on either of the Rib^- mutants, 64 out of 64 Em^r Lm^r transductants tested required riboflavin.

(ii) CP-51 was propagated on Bacillus anthracis UM23 Ura^- and the Pur^- and Rib^- mutants were transduced with selection for Ade^+ or Rib^+ transductants. These were then tested for sensitivity to Em and Lm. All of 56 Ade^+ transductants, all of 25 Rib^+ plus transductants of one riboflavin mutant, and all of 43 Rib^+ plus transductants of the other riboflavin mutant were sensitive to the antibiotics. We do not know whether the two Rib^- mutants are siblings or whether they represent independent mutations.

These experiments show that transpositions of Tn917 from pTV1 to the chromosome do occur in Bacillus anthracis and that pTV1 can be used as a tool to obtain insertion mutations.

Use of Tn917 for mutagenesis of pXO1. The mutagenesis procedure described has also been carried out on Weybridge A UM23(pXO1). I know of no way to select specifically cells in which the transposon has been inserted into the plasmid, and thus the identification of plasmid insertions is much more difficult than the identification of chromosomal insertions in a plasmid-free strain. The method we use to identify plasmid insertions consists of hybridization tests with ^{32}P-labeled Tn917 DNA.

The ease with which we isolated our first example of a cell carrying pXO1::Tn917 suggests that the transposon may favor the plasmid as a target. However, such a conclusion can not be made until we have isolated more examples. We currently have a number of other candidates to test and we should have the results shortly.

Our preliminary results indicate that our strain carrying the tagged pXO1 produces the same amount of protective antigen as the parent strain. It also sporulates in a manner resembling that of pXO1-containing strains, rather than strains cured of pXO1. We are currently testing the strain with pXO1::Tn917 for phage sensitivity and for rate of growth on minimal medium, two other characteristics that are influenced by pXO1.

Transductional shortening of Tn917-tagged pXO1. We have demonstrated that the phenomenon of transductional shortening can be applied to Tn917-tagged pXO1. Phage CP-51 that was grown on a strain carrying pXO1::Tn917 was used to transduce Weybridge A UM 3-2(pXO1)$^-$ with selection for resistance to Em and Lm. In the first and only experiment of this kind thus far carried out we obtained one transductant. (The frequency of transductional shortening would be expected to be quite low). It harbors a plasmid which appears to be roughly half the size of pXO1. It has been estimated that the CP-51 phage head can hold about 60 megadaltons of DNA, which is about half the size of pXO1.

In a test for protective antigen production in broth culture, the strain with the shortened version of pXO1 gave negative results. In the same experiment the original strain carrying pXO1::Tn917 produced a normal amount of protective antigen. We are currently testing for other phenotypic changes in the strain carrying the shortened version of pXO1.

If Tn917 inserts randomly in pXO1, it should be possible to isolate a large variety of shortened pXO1 plasmids. The availability of a collection of deleted plasmids would be of considerable help in studying the biology of pXO1.

IV. Further physical and genetic characterization of phage TP-21

For the past several years the plasmids of B. thuringiensis have been extensively studied due largely to an interest generated in plasmids encoding synthesis of insecticidal parasporal crystal and/or fertility functions. The 29-megadalton plasmid of B. thuringiensis subsp. kurstaki HD-1 was once thought by other workers to encode such functions. Our interest in the fertility plasmids of B. thuringiensis as tools for studying the genetics of B. anthracis has led us to the discovery that the 29-megadalton plasmid is the prophage of a bacteriophage we have designated TP-21. Although there are several examples in Gram-negative bacteria of phage having a plasmid prophage, to our knowledge there are no reports of such an occurrence in Gram-positive bacteria. One feature of the biology of TP-21 which we are interested in is the ability of TP-21 to carry the macrolide-lincosamide-streptogramin B (MLS) resistance transposon Tn917 and still retain infectivity. A TP-21 mutant which carries Tn917 and is easily cured would be a useful vector for transposon mutagenesis within B. anthracis, B. cereus and B. thuringiensis.

Lysogeny of B. thuringiensis subsp. kurstaki. Cell-free filtrates of B. thuringiensis subsp. kurstaki HD1-9 cultures contained greater than 1×10^9 TP-21 plaque-forming units per ml. Maximum titers were obtained when cultures were grown in brain heart infusion broth for 16-18 hours at 30°C. Phage lysates were assayed on soft agar lawns seeded with spores of B. cereus 569. Plaques from wild-type phage were turbid and indistinct; however, several factors have been found to improve plaquing: (i) Soft agar should contain only 0.3% agar; (ii) Plaques should be scored after 14 to 16 hours of incubation at 30°C; (iii) NBY medium containing 0.1% glycerol should be used; (iv) Exposure of assay plates to chloroform vapor makes the plaques clear and more distinct (Fig. 8).

Cells picked from the centers of plaques were lysogens and released phage having the same plaque morphology, although occasionally clear-plaque

mutants were found. The lysogens were immune to lysis by clear-plaque mutants. Cells lysogenized by the phage contained a newly acquired plasmid co-migrating with the 29-megadalton plasmid of B. thuringiensis subsp. kurstaki HD-1 (Fig. 9).

Transduction by TP-21. Cell-free lysates were effective in transferring chromosomal markers (Table 5). Plate transductions in which 0.1 ml of early stationary phase B. cereus recipient cells were spread on minimal medium along with 0.1 ml of cell-free lysate yielded up to 4×10^3 prototrophic transductants per ml of lysate. Cell-free lysates were also effective in transferring the tetracycline resistance plasmid pBC16 from tetracycline-resistant donors to tetracycline-sensitive recipients (Table 5). The number of tetracycline-resistant transductants ranged from 80 to 1000 per ml of lysate.

Comparison of phage DNA and plasmid DNA. DNA extracted from phage particles was linear and double stranded and slightly greater than 48.5 kb in length. The prophage is a covalently closed circular plasmid containing approximately 46 kb of DNA (Fig. 10). Restriction digests of DNA extracted from phage particles contained all fragments represented in digests of the plasmid prophage (data not shown). These features indicate that the phage DNA is circularly permuted and terminally redundant.

Phage DNA digested by the restriction enzyme PstI generated a single additional faint band, approximately 7.2 kb, not present when plasmid DNA was digested by this enzyme. This band may represent the end of concatemeric DNA resulting from a unique packaging origin in the phage DNA. Analogous results are documented for coliphage P1 (7).

Isolation of TP-21 carrying Tn917. B. cereus carrying pLS20::Tn917 was lysogenized with TP-21 and transposition was induced with a subinhibitory concentration of erythromycin (0.1 µg/ml). Phage to which Tn917 had transposed was isolated from filtrates of cultures thus induced. The culture filtrates were mixed with recipient cells and a sufficient time, 15 to 30 min, was allowed for phenotypic expression of erythromycin resistance. The mixture was combined with soft L agar containing an inducing concentration of erythromycin and then poured on plates of L agar containing selective concentrations of erythromycin and lincomycin. The erythromycin-resistant lysogens (transductants) were found to be immune to lysis by TP-21c-1, a clear-plaque mutant of TP-21, and to contain newly-acquired plasmid DNA. Although some transductants were defective lysogens unable to produce any infectious phage,

others contained phage which retained infectivity. Newly formed lysogens picked from the centers of plaques generated by lysates of these TP-21-producing erythromycin-resistant isolates were always found to be erythromycin resistant. Restriction digests of prophage DNA from the lysogens confirmed the presence of Tn917 (Fig. 11). AvaI does not cut TP-21 DNA but cleanly excises Tn917 sequences in three characteristic fragments. EcoRI does not cut Tn917 but cleaves TP-21 into several fragments. All of the defective prophages tested had some phage sequences deleted. Under selective pressure, lysogens of wild-type TP-21 were able to act as recipients of TP-21::Tn917 although at a lower frequency than the same recipient not carrying TP-21. Defective TP-21::Tn917 could be transferred from defective lysogens to recipient cells by transduction with phage CP-51. When the recipient cells were wild-type TP-21 lysogens and transductions were carried out under selective pressure for MLS resistance, defective TP-21::Tn917 could replace the wild-type phage from lysogens, including the natural host B. thuringiensis subsp. kurstaki HD1-9.

Restriction mapping of TP-21 and TP-21::Tn917 isolates. TP-21 phage and prophage DNA were digested with several restriction endonucleases and electrophoresed in agarose gels with concentrations of agarose varying from 0.3 to 1.2%. Single, double and triple digests using BglII, PstI and AvaI were run. The sizes of the restriction fragments are listed in Table 6. The sizes were estimated by comparing the relative migrations of restriction fragments of TP-21 and TP-21::Tn917 to the relative migrations of standard fragments.

The sizes of the prophage DNA from the defective TP-21::Tn917 isolates TP-21::Tn917-12 (app. 37 kb), TP-21::Tn917-13 (app. 26 kb) and TP-21::Tn917-23 (app. 42 kb) are such that a deletion of phage sequences must have occurred in addition to insertion of Tn917 which contains 5,257 bp. We have been able to determine the order of the restriction fragments relative to each other by comparing the restriction fragments of the defective prophage DNAs to those of the wild-type prophage. The alteration of two fragments by insertion of the transposon and subsequent deletion of phage sequences from the TP-21::Tn917 prophage points to adjacent positions of the two fragments on wild-type map. Any missing fragments would occupy a position on the wild-type map between those which have been altered. The results of mapping these restriction fragments are shown in Fig. 12.

The isolate TP-21::Tn*917*-13 contained the most extensive deletion, retaining only those sequences found on the largest *Pst*I fragment of the wild-type DNA. Each of the other two TP-21::Tn*917* isolates also contains most or all of this fragment. This, therefore, localizes the essential region for plasmid maintenance somewhere on this fragment.

TP-21 mutants. To isolate temperature-inducible mutants of TP-21 a lysate prepared in the presence of NTG was assayed at a high temperature. This allowed temperature inducible mutants to form plaques which appeared clear. Several mutants were isolated which made clear-plaques at a high temperature and these were tested at a low temperature. Four isolates designated by the appendant notation c-3, c-7, c-8 and c-12 formed plaques which were less clear at 30°C then at 42°C. Unlike true clear-plaque mutants these isolates could lysogenize *B. cereus* 569 at a high multiplicity of infection. These four TP-21 mutants were tested for thermoinducibility. TP-21c-12 appeared to be significantly temperature inducible. Assays of culture filtrates of TP-21c-12 lysogens grown at 42°C revealed a 30-fold higher phage titer than a filtrate of a similar culture grown at 30°C. The 42°C cultures of lysogens of the other isolates had either a lower titer compared to the 30°C control or only a small increase.

Scott has found that a conditional clear-plaque mutant of *E. coli* phage P1, P1 *c7*, lysogenized cells at low temperatures but the lysogens were cured of the phage when they were shifted to a high temperature (24). With this in mind we tested lysogens of the isolates TP-21c-3, TP-21c-7, TP-21c-8 and TP-21c-12. The lysogens were grown at 42°C in broth and then streaked onto NBY agar plates. Colonies were tested for the presence of TP-21 prophage by picking 50 representative colonies to soft agar lawns of *B. cereus* 569. Colonies from the heat-treated cultures of the lysogens carrying TP-21c-7 and TP-21c-12 made no zones of clearing in the lawns. These results suggested that these two mutants were temperature sensitive for replication and were especially good candidates for use in creating a selection vector for Tn*917* mutagenesis.

All of the clear-plaque mutants isolated following NTG mutagenesis were tested for their ability to complement the clear-plaque mutation of TP-21c-1, a spontaneous clear-plaque mutant. Soft agar lawns seeded with *B. cereus* 569 spores were spotted with phage lysate, one spot contained TP-21c-1, the second spot contained the test clear-plaque mutant, and the third spot contained a 1:1 mixture of TP-21c-1 and the test mutant. In these tests the 4 mutants,

TP-21c-3, TP-21c-7, TP-21c-8 and TP-21c-12, whose plaques were less clear than those of TP-21c-1, did complement TP-21c-1. Another mutant, TP-21c-2, which produced large crystal-clear plaques like those produced by TP-21c-1, could also complement TP-21c-1. These results suggest there are at least two cistrons involved in lysogeny.

TP-21 transposition selection vector. We have isolated derivatives of TP-21c-7 containing a copy of Tn*917*. As reported above, TP-21c-7 was found to be temperature curable in *B. cereus* 569. Cell-free lysates containing TP-21c-7::Tn*917* were able to transfer MLS resistance to both *B. anthracis* and *B. cereus*. With *B. cereus* this phage vector formed lysogens; however, with the Weybridge strain of *B. anthracis* it appeared to behave as a suicide vector. MLS-resistant transductants of Weybridge UM23 did not carry any plasmids other than pXO1 originally present in the recipients. It seems possible that this instability of TP-21c-7::Tn*917* in *B. anthracis*, resulting in a suicide vector, might render this derivative useful for convenient transposon mutagenesis. Such a vector would achieve essentially the same goal as a vector temperature sensitive for replication in that Tn*917* could be delivered to target sequences without the vector remaining. The effectiveness of this derivative in mutagenesis of *B. anthracis* will depend on its ability to infect cells at a high frequency; this remains to be determined. If our preliminary observations on the temperature sensitivity of TP-21c-7 in *B. cereus* 569 are confirmed with its Tn*917* derivative, then this mutant should be a useful vector for transposon mutagenesis in that strain. We could then transfer the *B. anthracis* plasmids to *B. cereus* for mutagenesis.

V. Further characterization of conjugative plasmid pLS20

The recent discovery that the *Bacillus subtilis* (*natto*) plasmid pLS20 encodes functions that allow conjugal plasmid transfer to occur was of interest to our laboratory for several reasons. This was the first documentation of a fertility plasmid in a species of *Bacillus* other than *B. thuringiensis*. This plasmid could be transferred by a mating event from *B. subtilis* (*natto*) into genetically similar strains of *Bacillus* at reasonably high frequencies as well as into the genetically distinct species *B. anthracis*, *B. cereus*, and *B. thuringiensis* at low frequencies. The ability of pLS20 to promote the trans-

fer of small antibiotic-resistance plasmids as well as the transfer of itself across these species may be important for the genetic analysis of cloned DNA sequences originating from the latter group of bacilli. This fertility plasmid should allow us to transfer cloned DNA from B. subtilis to B. anthracis for phenotypic characterization.

Thus, it is important for us to characterize pLS20 at the molecular level, i.e., determine the size and location of the transfer genes, so that we may be able to determine the factors involved in the mechanism of conjugal DNA exchange among Bacillus species and potentially create a self-mobilizable cloning vehicle.

Research on pLS20 during the past year has focused upon the following:

1. Investigation of whether pLS20 DNA exhibits any homology with pXO12 or pAMβ1 DNA.
2. More precise sizing of pLS20 utilizing several restriction enzymes and a range of agarose concentrations.
3. Generation of Tra^+ and Tra^- Tn917-tagged pLS20 derivatives.
4. Restriction analysis of pLS20 and derivatives to localize particular fragments of DNA implicated in conjugal plasmid transfer.
5. Analysis of deletion derivatives of pLS20::Tn917 plasmids.

Test for homology of pLS20 with other fertility factors. Hybridization experiments were carried out to determine the extent of homology, if any, between the B. subtilis (natto) fertility plasmid pLS20 and the B. thuringiensis fertility plasmid pXO12 or the streptococcal fertility plasmid pAMβ1. DNA extracted from B. subtilis 838(pAMβ1), B. cereus UM20-1(pXO12), and B. anthracis Weybridge A UM23-23(pLS20) was digested with the restriction endonucleases BglII and EcoRI and electrophoresed on agarose gels. The DNA was transferred to GeneScreen Plus membranes and probed with ^{32}P-labelled pXO12 or pAMβ1 DNA. In each case the probe hybridized only to itself; no homology was observed between pLS20 and pAMβ1 or pXO12.

Size determination of pLS20. Previously the size of pLS20 was reported to be 55 kb (T. Koehler, PhD thesis, Univ. of Massachusetts, 1987). However, more extensive analysis has revealed that the size of the plasmid is actually larger. The size was determined by digestion of the plasmid DNA with a total of five restriction endonucleases. The resulting digestion mixtures were then electrophoresed on agarose gels ranging in concentration from 0.3% to 1.2%. The size estimates for pLS20 ranged from 61.4 kilobases (with EcoR1) to 65.0

kilobases (with Pst1). The average size of pLS20 was calculated to be 64 kilobases. The number and sizes of restriction fragments obtained with 4 of the 5 enzymes used are shown in Table 7. Restriction of pLS20 with EcoR1 generated a total of 27 fragments ranging in size from 7.25 kilobases to 0.43 kilobases and the results are not shown here.

Isolation of Tn917-tagged pLS20 derivatives. One approach utilized to localize the region(s) of pLS20 necessary for plasmid-mediated conjugal DNA transfer was to generate insertions of Tn917 in pLS20 and determine the effects of the insertions on conjugation. A total of 13 novel Tn917 insertions into pLS20 are presently available for analysis. These were generated and identified by three different methods. The transposon-tagged plasmids pXO501 through pXO504 were isolated by T. Koehler by a procedure which involved transfer of pLS20::Tn917 from a culture of B. subtilis(pLS20, pTV1) that had been induced for transposition and used as a donor of MLS resistance. (It had already been shown that pLS20 was very inefficient, if not completely inactive, in mobilizing pTV1). A prerequisite for the isolation of these plasmids was that the insertion of Tn917 did not disrupt any gene(s) necessary for self-mobilization.

The next procedure was a random approach in which B. subtilis PSL1 UM3(pTV1, pLS20, pBC16) was grown in broth containing erythromycin (0.1 µg/ml) to induce transposition and then transferred to 45°C to eliminate pTV1. Cells were then streaked on L agar containing selective levels of erythromycin and lincomycin. Colonies were picked to L agar with erythromycin and L agar with chloramphenicol to screen for those cells no longer harboring pTV1 but still resistant to the MLS antibiotics. Plasmid lysates from transposants detected in this manner were electrophoresed on agarose gels and hybridizations were carried out to determine the genomic location of Tn917. The probe was obtained by digesting pTV1 DNA with AvaI, eluting the Tn917 fragments from an agarose gel, and radiolabelling the fragments with ^{32}P-dGTP to provide a specific probe for the transposon. Once the location of Tn917 had been shown to be on pLS20, the tagged plasmids were analyzed by restriction endonuclease digestion with BglII. The restriction analysis revealed 6 novel Tn917-tagged plasmids, and these were denoted pXO505 through pXO510. However, since these six transposants were not derived from six independent cultures we can not be sure that they represent independent transposition events.

The third procedure for isolating transposon-tagged plasmids has overcome the problems encountered with the first two procedures. With this procedure, each tagged plasmid is the result of both a random and an independent transposition event. The procedure was the same as that just described except that only one chloramphenicol-sensitive MLS-resistant colony from a particular culture was subjected to further analysis. Among a total of 31 independent pLS20::Tn*917* plasmids isolated in this manner we found three new Tn*917* insertions, designated pXO511 through pXO513. The remaining 28 tagged plasmids resembled the previously isolated plasmids pXO503 through pXO510.

Transfer phenotypes of cells harboring Tn*917*-tagged plasmids. The *B. subtilis* isolates harboring the pLS20::Tn*917* plasmids pXO501 through pXO512 were used as donors in membrane matings to determine the transfer phenotype conferred on the host by each of the tagged plasmids. The results are summarized in Table 10 which shows the transfer phenotypes of cells harboring each of the plasmids. Table 10 also lists sizes of the *Bgl*II restriction fragments generated from the plasmids.

B. subtilis cells containing pXO501 transferred the fertility plasmid at somewhat reduced efficiency, while cells carrying pXO502, pXO503, or pXO504 appeared to possess plasmid transfer ability essentially unaltered from that of cells carrying wild-type pLS20 (Cells harboring pXO501 and pXO502 were not tested for the ability to donate pBC16). Among the plasmids pXO505 through pXO512 three, pXO505, pXO509, and pXO510, appeared to be practically incapable of conferring fertility for self transfer or mobilization of pBC16. The insertions in these plasmids may directly affect a gene encoding a nuclease that is responsible for introducing a nick into plasmid DNA that is to be transferred. Cells harboring pXO506, pXO507, or pXO508 transferred pBC16 at reduced frequencies and the fertility plasmid at almost negligible frequencies. pXO511, which promoted transfer of pBC16 at a high frequency, was apparently unable to promote its own transfer. This derivative may have the transposon located in a region of the plasmid necessary for self-transfer. Most of the derivatives that were defective in mobilizing themselves contain the transposon within the 10.8-kb *Bgl*II fragment (pXO511 contains an insertion within the 14.4-kb *Bgl*II fragment), whereas those derivatives capable of self-transfer all contain insertions within the 14.4-kb or 27-kb *Bgl*II fragments. The derivatives apparently unaffected in either self-mobilization or the mobilization of pBC16 contain insertions within the 27.0-kb *Bgl*II fragment.

Therefore, the procedures employed for generating Tn917-tagged derivatives were successful in that both Tra^+ and Tra^- derivatives were isolated. The transfer phenotypes can be seen in Table 10 and correlated with the locations of the transposon insertions.

Formation of deletions. Growth of cells carrying certain Tn917-tagged pLS20 derivatives, namely pX0503 and pX0504, for several generations in the presence of selective levels of erythromycin (1 μg/ml) led to the formation of deletant plasmids. This phenomenon has been demonstrated to occur in B. subtilis and in B. anthracis. None of the other transposon-tagged derivatives appeared to undergo deletions when cells carrying them were grown under the same conditions. pX0501, pX0502, and pX0505 through pX0512 appeared unchanged after host cells were grown in the presence of erythromycin, showing no alterations upon digestion with the restriction endonucleases BglII and AvaI. It is possible that these plasmids also incur deletions during growth of host cells in the presence of erythromycin, but the deletions might encompass one or more genes necessary for plasmid maintenance or replication.

The approximately 15 kb of pLS20 DNA missing from the deletants of pX0503 and pX0504 described above rendered the host cells transfer-deficient. B. subtilis cells and B. anthracis cells harboring these deletant plasmids were incapable of transferring either the fertility plasmid or pBC16 in membrane matings.

TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain or plasmid	Relevant characteristics[a] and plasmids	Origin or Reference[b]
B. anthracis		
Weybridge	Avirulent, Tox^+ Cap^- pXO1	27
Weybridge UM44	Ind^- Tox^+ Cap^- pXO1	UV of Weybridge
Weybridge UM44-1	Ind^- Tox^+ Str^r, pXO1	1
Weybridge UM44-2 (same as UM44-1C9)	Ind^- Tox^- Str^r, $(pXO1)^-$	Curing of UM44-1
Weybridge UM44-4	Ind^- Tox^+ Str^r Cry^+ Tra^+ Tc^r, pXO1, pXO12, pBC16	UM44-1 tr203-23 (1)
Weybridge UM44-5	Ind^- Tox^- Str^r Cry^+ Tra^-, pXO12-4 $(pXO1, pBC16)^-$	Curing of UM44-4
Weybridge UM44-6	Ind^- Tox^+ Str^r Cry^+ Tc^r, pXO1-2, pXO12, pBC16	A UM2-3 x UM44-2
Weybridge UM44-7	Ind^- Tox^+ Str^r Cry^-, pXO1-2, $(pXO12, pBC16)^-$	Curing of UM44-6
Weybridge UM44-8	Ind^- Tox^+ Str^r Cry^+ Tc^r, pXO1-4, pXO12, pBC16	A UM2-3 x UM44-2
Weybridge UM44-9	Ind^- Tox^+ Str^r Cry^+ Tc^r, pXO1::pXO12, pBC16	A UM17-3 x UM44-2
Weybridge UM44-10	Ind^- Tox^- Str^r Cap^+ MLS^r, pXO2-8	6602 UM5 x UM44-2
Weybridge UM44-11	Ind^- Tox^- Str^r Cry^+ Cap^+ MLS^r Tc^r, pXO2-8, pXO12, pBC16	A UM23-8 x UM44-10
Weybridge A	Colony variant of Weybridge, Tox^+, pXO1	27
Weybridge A UM2	Trp^- Tox^+, pXO1	UV of Weybridge A

(Table 1 continued)

Weybridge A UM2-3	Trp$^-$ Tox$^+$ Cry$^+$ Tc^r, pXO1-1, pXO12, pBC16	A UM23-20 x A UM2
Weybridge A UM2-4	Trp$^-$ Tox$^+$ Cry$^-$, pXO1-1, (pXO12, pBC16)$^-$	Curing of A UM2-3
Weybridge A UM17	Ade$^-$ Tox$^+$, pXO1	UV of Weybridge A
Weybridge A UM17-3	Ade$^-$ Tox$^+$ Cry$^+$ Tc^r, pXO1, pXO12, pBC16	A UM17 tr57B-6 (1)
Weybridge A UM17-4	Ade$^-$ Tox$^+$ Cry$^+$ Tc^r, pXO1, pXO12, pBC16	A UM23-20 x A UM17
Weybridge A UM18-5	pyrA1 Tox$^-$ Str^r, (pXO1)$^-$	C. B. Thorne
Weybridge A UM18-6	pyrA1 Tox$^+$ Str^r Cry$^+$ Tra$^+$ Tc^r, pXO1, pXO12-3, pBC16	UM44-6 x A UM18-5
Weybridge A UM18-7	pyrA1 Tox$^-$ Str^r Cry$^+$ Tra$^-$, pXO12-3	Curing of A UM18-6
Weybridge A UM23	Ura$^-$ Tox$^+$ pXO1	UV of Weybridge A
Weybridge A UM23-1	Ura$^-$ Tox$^+$ pXO1 Str^r	UV of UM23
Weybridge A UM23-2 (same as A UM23C1)	Ura$^-$ Tox$^-$ (pXO1)$^-$	Curing of A UM23
Weybridge A UM23-3 (same as A UM23C1-1)	Ura$^-$ Tox$^-$ Str^r, (pXO1)$^-$	UV of A UM23-2
Weybridge A UM23-4 (same as A UM23C1-2)	Ura$^-$ Tox$^-$ Rif^r, (pXO1)$^-$	UV of A UM23-2
Weybridge A UM23-5	Ura$^-$ Tox$^-$ Rif^r MLS^r Tra$^+$, pXO503	10
Weybridge A UM23-6	Ura$^-$ Tox$^-$ Cap$^+$ Str^r, pXO2-1	td of pXO2-1 from B. cereus 569 UM20-6 to A UM23-3
Weybridge A UM23-7	Ura$^-$ Tox$^-$ Cap$^+$ Str^r, pXO2-2	td of pXO2-2 from B. cereus 569 UM20-2 to A UM23-3

(Table 1 continued)

Weybridge A UM23-8	Ura$^-$ Tox$^-$ Rif^r Cry$^+$ Tc^r, pXO12, pBC16	A UM2-3 x A UM23-4
Weybridge A UM23-9	Ura$^-$ Tox$^+$ Rif^r Tc^r, pXO1-3, pBC16	A UM2-3 x A UM23-4
Weybridge A UM23-10	Ura$^-$ Tox$^+$ Rif^r, pXO1-3, (pBC16)$^-$	Curing of A UM23-9
Weybridge A UM23-11	Ura$^-$ Tox$^+$ Rif^r Cry$^+$ Tc^r, pXO1-5, pXO12, pBC16	A UM2-3 x A UM23-4
Weybridge A UM23-12	Ura$^-$ Tox$^+$ Rif^r Cry$^+$ Tc^r, pXO1-1::pXO12^c, pBC16	A UM2-3 x A UM23-4
Weybridge A UM23-13	Ura$^-$ Tox$^+$ Rif^r Cry$^+$ Tc^r, pXO1-6, pXO12, pBC16	A UM2-3 x A UM23-4
Weybridge A UM23-14	Ura$^-$ Tox$^+$ Rif^r Cry$^+$ Tc^r, pXO1-7, pXO12, pBC16	A UM17-4 x A UM23-4
Weybridge A UM23-15	Ura$^-$ Tox$^+$ Rif^r Cry$^+$ Tc^r, pXO1, pXO12, pBC16	A UM17-4 x A UM23-4
Weybridge A UM23-16	Ura$^-$ Tox$^-$ Rif^r Cry$^+$ Tc^r MLS^r, pXO12, pXO503, pBC16	UM44-4 x A UM23-5
Weybridge A UM23-18	Ura$^-$ Tox$^-$ Rif^r Cry$^-$ Tra$^+$ MLS^r, pXO12-1	B. cereus 569 UM20-12 x A UM23-4
Weybridge A UM23-20	Ura$^-$ Tox$^-$ Cry$^+$ Tra$^+$ Tc^r, pXO12, pBC16	A UM23C2 tr237-10 (1)
Weybridge A UM23-21	Ura$^-$ Tox$^-$ Cry$^+$ Tra$^+$ Tc^r, pXO12, pBC16	A UM23C2 tr60B-1 (1)
Weybridge A UM23-22	Ura$^-$ Tox$^-$ Str^r Tc^r, pLS20, pBC16	PSL1 UM3 x A UM23-3
Weybridge A UM23-23	Ura$^-$ Tox$^-$ Str^r, pLS20, (pBC16)$^-$	Curing of A UM23-22
4229 (Pasteur)	Cap$^+$ Tox$^-$, pXO2	6
4229 UM12	Cap$^+$ Nal^r, pXO2	UV of 4229
4229 UM12-1	Cap$^+$ Nal^r Cry$^+$ Tc^r, pXO2, pXO12, pBC16	4229 UM12 tr299-3 (6)
4229 UM12-2	Cap$^+$ Nal^r Cry$^+$ Tc^r, pXO2, pXO12, pBC16	B. cereus 569 UM20-5 x 4229 UM12
6602 (Pasteur)	Cap$^+$ Tox$^-$, pXO2	6

(Table 1 continued)

6602 UM4	Cap$^+$ Cry$^+$ Tc^r, pXO2, pXO12^d, pBC16	A UM23-21 x 6602
6602 UM5	Cap$^+$ MLS^r, Cry$^-$ Tra$^+$, pXO2, pXO12-2	B. cereus 569 UM20-13 x 6602
B. cereus		
569	Wild type	NRRL
569 K	Trp$^-$ Str^r	A. Aronson
569 UM20-1	Ant$^-$ Str^r	1
569 UM20-2	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-2, pXO12, pBC16	6602 UM4 x 569 UM20-1
569 UM20-3	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-3, pXO12, pBC16	6602 UM4 x 569 UM20-1
569 UM20-4	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2::pXO12^e, pBC16	6602 UM4 x 569 UM20-1
569 UM20-5	Ant$^-$ Str^r Cry$^+$ Tc^r, pXO12, pBC16	A UM17-4 x 569 UM20-1
569 UM20-6	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-1, pXO12, pBC16	4229 UM12-1 x 569 UM20-1
569 UM20-7	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-4, pXO12, pBC16	4229 UM12-1 x 569 UM20-1
569 UM20-8	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2, pXO12, pBC16	4229 UM12-1 x 569 UM20-1
569 UM20-9	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-5, pXO12, pBC16	4229 UM12-2 x 569 UM20-1
569 UM20-10	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-6, pXO12, pBC16	4229 UM12-2 x 569 UM20-1
569 UM20-11	Ant$^-$ Str^r Cap$^+$ Cry$^+$ Tc^r, pXO2-7, pXO12, pBC16	4229 UM12-2 x 569 UM20-1
569 UM20-12	Ant$^-$ Str^r MLS^r Cry$^-$ Tc^r Tra$^+$, pXO12-1, pBC16	A UM23-16 x 569 UM20-1
569 UM20-13	Ant$^-$ Str^r MLS^r Cry$^-$ Tra$^+$, pXO12-2	A UM23-16 x 569 UM20-1
569 UM24-8	Met$^-$ Leu$^-$ Str^r	C. Thorne

(Table 1 continued)

569 UM26-10	Met$^-$ Ile$^-$ Str^r	C. Thorne
B. subtilis		
838	pAMβ1	S. Zahler
PSL1	r$^-$ m$^-$ Arg$^-$ Thr$^-$ Leu$^-$	BGSC strain 1A510
PSL1 UM3	r$^-$ m$^-$ Arg$^-$ Thr$^-$ Leu$^-$ pLS20, pTV1, pBC16	T. Koehler
B. thuringiensis		
HD1-9	subsp. *kurstaki*, carries phage TP-21	A. Aronson
Bacteriophages		
CP-51	Generalized transducing phage	C. B. Thorne
CP-51*ts*45	Temperature-sensitive mutant of CP-51	C. B. Thorne
TP-21	Prophage is a plasmid	*B. thuringiensis* subsp. *kurstaki* HD1-9
Plasmids		
pBC16	Tc^r	2
pLS20	Conjugative plasmid of *B. subtilis* (*natto*)	10
pXO1	Encodes synthesis of *B. anthracis* toxin, Tox$^+$	19, 27
pXO1-1	pXO1 with 2.6-kb insert from pXO12, Tox$^+$	This study
pXO1-2 to pXO1-6	pXO1-1::Tn*4430*, Tox$^+$	This study
pXO1-7	pXO1::Tn*4430*, Tox$^+$	This study
pXO2	Encodes synthesis of *B. anthracis* capsule, Cap$^+$	6

(Table 1 continued)

pXO2-1 to pXO2-7	pXO2::Tn*4430*, Cap^+	This study
pXO2-8	pXO2::Tn*917*, Cap^+	This study
pXO12	Tra^+ Cry^+ from *B. thuringiensis*	1
pXO12-1 and pXO12-2	pXO12::Tn*917*, Tra^+ Cry^-	This study
pXO12-3 and pXO12-4	Deletion derivatives of pXO12, Tra^- Cry^+	This study
pXO501 to pXO503	pLS20::Tn*917*	10
pXO504 to pXO512	pLS20::Tn*917*	This study

Abbreviations: Ade, adenine; Ant, anthranilic acid; Cry, synthesis of parasporal crystal; Ind, indole; Trp, tryptophan; Ura, uracil; MLS^r, Tn*917*-encoded macrolide, lincosamide, and streptogramin B resistance; Nal^r, naladixic acid resistance; Rif^r, rifampicin resistant, Str^r, streptomycin resistant; Tc^r, pBC16 encoded tetracycline resistance; Cap, synthesis of polyglutamate capsule; Tox, synthesis of protective antigen component of the anthrax toxin; Tra, mediation of plasmid transfer by mating.

[b] BGSC, *Bacillus* Genetics Stock Center, Columbus, Ohio; NRRL, Northern Regional Research Laboratory, Departament of Agriculture, Peoria, IL; UV, mutagenesis by UV light (26). td, phage CP-51-mediated transduction (23). Matings are shown as donor strain x recipient strain.

[c] A presumed cointegrate of pXO1-1 and pXO12.

[d] pXO12 in 6602 UM4 is an uncharacterized deletion derivative.

[e] A presumed cointegrate of pXO2 and pXO12.

TABLE 2. Mobilization of *B. anthracis* plasmids pXO1 and pXO2 by pXO12.

Expt	*B. anthracis* Weybridge		Tc^r transcipients		MLS^r transcipients	
	Donor	Recipient	No. per ml	% Tox^{+a}	No. per ml	% Cap^{+b}
A	A UM17-4(pXO1, pXO12, pBC16)	A UM23-4	2.0×10^5	0.27 (2/742)	NA[c]	NA
	A UM2-3(pXO1-1, pXO12, pBC16)	UM44-2	9.1×10^3	0.42 (2/475)	NA	NA
B	UM44-11(pXO2-8, pXO12, pBC16)	A UM23-4	2.2×10^5	NA	2.7×10^3	45 (45/100)
	UM44-11(pXO2-8, pXO12, pBC16)	A UM23-4	1.9×10^5	NA	4.0×10^2	46 (44/95)

[a] Percentage of Tc^r transcipients which were Tox^+. Numbers in parentheses represent the number of Tox^+ transcipients over the number of Tc^r transcipients tested.

[b] Percentage of MLS^r transcipients which were Cap^+. Numbers in parentheses represent the number of Cap^+ transcipients over the number of MLS^r transcipients tested.

[c] Not applicable.

TABLE 3. Transfer of pXO1::pXO12 and pXO2::pXO12 cointegrate plasmids

Expt.	Donor strain	Recipient strain	Tc^r transcipients			
			No. per ml	Frequency[a]	% Tox^+Cry^+[b]	% Cap^+Cry^+[c]
A	Weybridge UM44-9 (pXO1::pXO12, pBC16)	Weybridge A UM23-4	4.0×10^5	1.4×10^{-2}	57 (495/874)	NA[d]
	Weybridge A UM23-12 (pXO1-1::pXO12, pBC16)	Weybridge UM44-2	6.8×10^2	2.1×10^{-4}	49 (83/170)	NA
B	B. cereus 569 UM20-4 (pXO2::pXO12, pBC16)	Weybridge A UM23-4	3.5×10^4	ND[e]	NA	24
	B. cereus 569 UM20-4 (pXO2::pXO12, pBC16)	Weybridge A UM23-4	1.4×10^4	1.7×10^{-4}	NA	17

[a] Frequency is expressed as the number of Tc^r transcipients per donor.

[b] Percentage of Tc^r transcipients which were Tox^+ Cry^+. Numbers in parentheses represent the number of Tox^+ Cry^+ transcipients over the number of Tc^r transcipients tested.

[c] Percentage of Tc^r transcipients which were Cap^+ Cry^+. Transcipients were selected on agar plates containing tetracycline and $NaHCO_3$ and incubated in 20% CO_2, allowing direct scoring of Tc^r Cap^+ colonies. All of 50 or more Tc^r Cap^+ transcipients tested were Cry^+. Similar numbers of Tc^r Cap^- transcipients were found tc be Cry^-.

[d] Not applicable.

[e] Not determined.

TABLE 4. Frequencies of pXO12-mediated transfer of pBC16 compared with transfer frequencies of pXO12::Tn*917* derivatives

Donor strain	Transcipients with recipient strain[a]			
	B. anthracis		*B. cereus*	
	No. per ml	Frequency	No. per ml	Frequency
B. anthracis Weybridge A UM23-20(pXO12,pBC16)	9.0×10^{5}	1.9×10^{-2}	3.9×10^{6}	7.1×10^{-2}
B. cereus 569 UM20-5 (pXO12,pBC16)	4.8×10^{4}	1.6×10^{-4}	ND[b]	ND
B. anthracis Weybridge A UM23-18(pXO12-1)	2.3×10^{5}	1.3×10^{-2}	4.5×10^{5}	2.6×10^{-2}
B. cereus 569 UM20-13 (pXO12-2)	2.2×10^{4}	9.2×10^{-5}	ND	ND

[a] Tc^{r} transcipients were selected when the donor strain contained pXO12 and pBC16. MLS^{r} transcipients were selected when the donor strain contained pXO12::Tn*917*, i.e., pXO12-1 and pXO12-2. Counterselection of donors was facilitated by the use of recipient strains that were resistant to streptomycin or rifampicin. Frequencies are expressed as the number of transcipients per donor.

[b] Not determined.

TABLE 5. Chromosomal and plasmid transduction of B. cereus 569 by TP-21

Donor lysate	Recipient	Transductants per/ml	Transductants per/PFU
B. thuringiensis HD1-9	B. cereus 569 UM26-10 Met^- Ile^- Str^r	4×10^3 (Ile^+)	9×10^{-6}
	B. cereus UM24-8 Met^- Leu^- Str^r	2×10^3 (Leu^+)	4×10^{-6}
	B. cereus 569 K Trp^- Str^r	3×10^3 (Trp^+)	2×10^{-5}
B. cereus 569 (TP-21)	B. cereus 569 UM26-10 Met^- Ile^- Str^r	5×10^2 (Ile^+)	1×10^{-6}
	B. cereus 569 K Trp^- Str^r	3×10^1 (Trp^+)	6×10^{-7}
B. anthracis 4229 (pBC16, TP-21)	B. cereus 569 K Trp^- Str^r	1×10^3 (Tc^r)	2×10^{-7}

Prototrophic transductants were selected on minimal medium. Tetracycline-resistant transductants, spread on membranes, were selected on L agar with tetracycline (25 µg/ml) after 5 hours for phenotypic expression on L agar. Controls in which peptone was substituted for lysate gave no prototrophic revertants or spontaneous tetracycline-resistant mutants.

TABLE 6. Restriction fragments of TP-21, TP-21::Tn917-12, TP-21::Tn917-13 and TP-21::Tn917-23[a]

	TP-21 DNA digested with:			TP-21::Tn917-12 DNA digested with:				
	PstI	PstI BglII	BglII	PstI	PstI AvaI	PstI BglII	PstI BglII AvaI	BglII
	24.0	9.4	19.6	25.4	18.5	9.4	9.4	17.8
	10.4	9.0	9.0	9.4	9.4	9.1	9.0	9.0
	9.4	8.5	8.5	2.5	3.0	8.5	8.5	8.5
	2.5	6.7	8.2		2.5	3.9	2.5	1.9
		5.4			1.7	2.5	2.2	
		4.6			1.3	2.2	1.7	
		2.5				1.9	1.5	
		2.3					1.2	
							1.0	
							0.8	
sum:	46.3	48.4	45.3	37.3	36.4	37.5	37.8	37.2

	TP-21::Tn917-13 DNA digested with:					TP-21::Tn917-23 DNA digested with:			
	PstI	PstI AvaI	PstI BglII	PstI BglII AvaI	BglII	PstI	PstI AvaI	PstI BglII	BglII
	26.1	20.0	8.5	9.0	8.5	24.0	24.0	8.5	9.5
		2.3	8.3	8.5	8.3	10.2	10.4	8.3	9.0
		1.7	4.6	2.2	4.6	8.0	2.5	6.5	8.5
		1.2	1.8	1.9	3.2		2.3	5.2	8.3
				1.7	1.8		1.7	4.6	5.3
				1.5			1.2	3.0	1.8
				1.0				1.9	
				0.8				1.8	
sum:	26.1	25.2	23.2	26.6	26.4	42.2	42.2	39.8	42.4

[a] Numbers represent fragment sizes in kilobases, underlined numbers indicate size of fragments contained entirely within Tn917.

TABLE 7. Restriction fragments generated from pLS20 with BglII, PstI, AvaI and StuI

AvaI		BglII	
Fragment	Size (kb)	Fragment	Size (kb)
1	24.6	1	27.0
2	18.6	2	14.6
3	11.6	3	10.8
4	6.85	4	6.5
5	3.2	5	5.4
Total	64.85		64.3

PstI		StuI	
Fragment	Size (kb)	Fragment	Size (kb)
1	14.4	1	46.8
2	12.8	2	14.4
3	6.3	3	2.95
4	6.15		
5	5.2		
6	4.4	Total	64.15
7	3.6		
8	3.4		
9	3.3		
10	3.15		
11	2.3		
Total	65.0		

TABLE 8. Comparison of transfer efficiency and BglII restriction patterns of pLS20 and derivatives

Plasmid	Effectiveness of transfer		BglII Restriction Fragments										
	Self	pBC16											
pLS20	+++	+++	27.0	14.4	10.8			6.5		5.4			
pXO501	++	ND	**24.0**	14.4	10.8			6.5	**6.4**	5.4		1.7*	
pXO502	+++	ND	27.0	**12.7**	10.8			6.5		5.4	**5.3**	1.7*	
pXO503	+++	+++	**28.0**	14.4	10.8			6.5		5.4	2.8*	1.7*	0.7*
pXO504	+++	+++	**28.0**	14.4	10.8			6.5		5.4	2.8*	1.7*	
pXO505	-	+/-	27.0	14.4		**7.3**	**6.8**	6.5		5.4		1.7*	
pXO506	+/-	+/++	27.0	14.4		**7.9**	**6.6**	6.5		5.4		1.7*	
pXO507	-	+/++	27.0	14.4		**7.1**	**7.0**	6.5		5.4		1.7*	
pXO508	-	++	27.0	14.4		**8.7**		6.5		5.4		1.7*	
pXO509	-	-	27.0	14.4		**8.5**		6.5		5.4	**2.6**	1.7*	
pXO510	-	-	27.0	14.4		**9.1**		6.5		5.4	**4.6**	1.7*	
pXO511	-	++++	27.0		10.8	**9.1**	**8.1**	6.5		5.4		1.7*	
pXO512	++	+++	**25.0**	14.4	10.8			6.5	5.8	5.4		1.7*	

New fragments resulting from the insertion of Tn917 are typed in boldface. The fragments representing Tn917 are denoted with an asterisk. The transfer phenotype conferred upon the host cell by the pLS20 derivative is separated into the processes of self-transfer and the transfer of pBC16. The values are compared to the transfer potential exhibited by the native fertility plasmid pLS20. +++, transfer comparable to native plasmid; ++, significant transfer but less than native pLS20; +, slight transfer; -, no transfer.

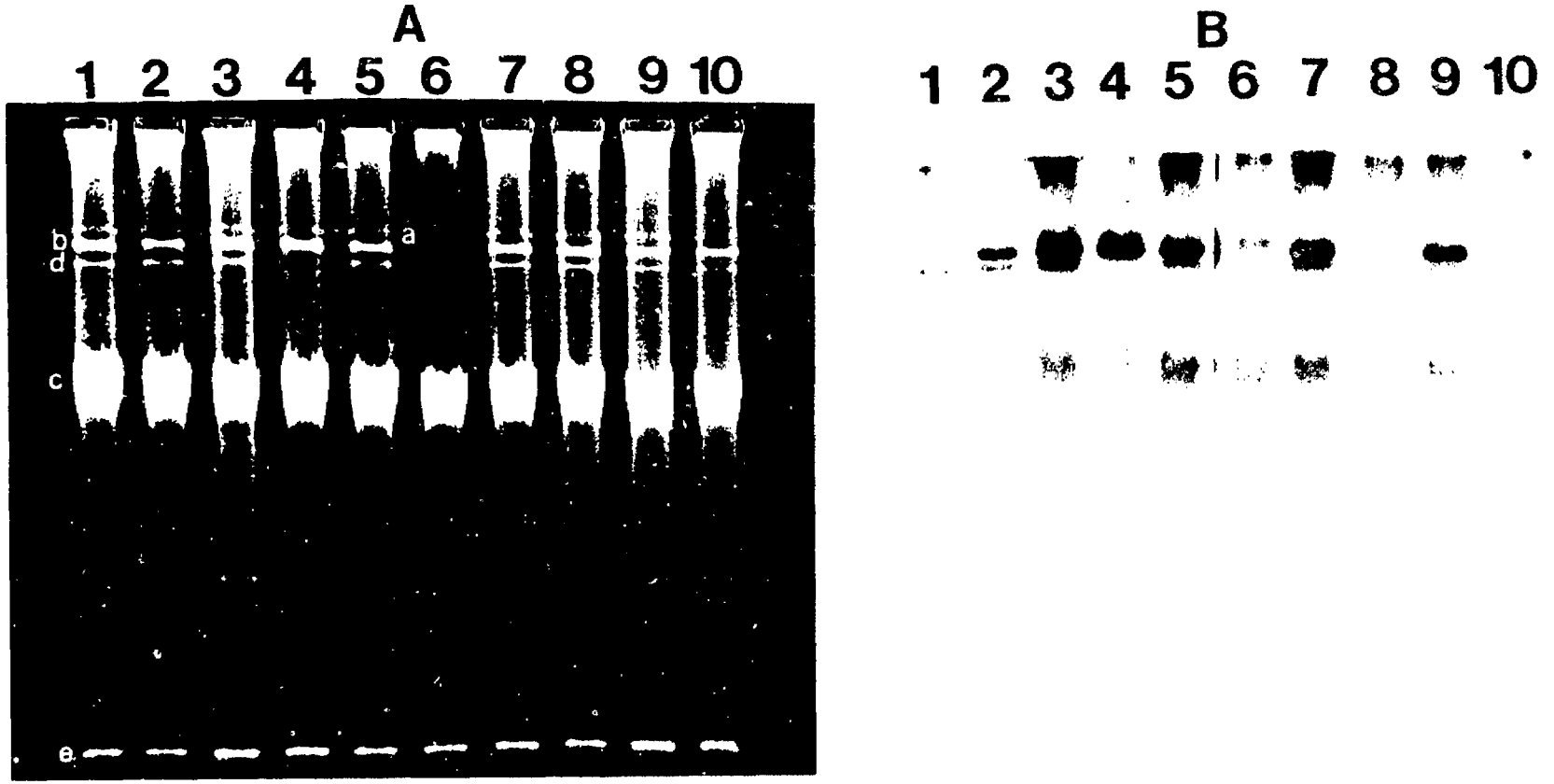

FIG. 1. Demonstration of Tn4430 transposition from pXO12 to pXO1 plasmids following their transfer by pXO12-mediated conjugation. Weybridge A UM2-3 was the donor for transcipients in lanes 2 through 7, and Weybridge A UM17-4 was the donor for transcipients in lanes 9 and 10. The recipients (not shown), Weybridge UM44-2 and Weybridge A UM23-4, contained no plasmids before mating. (A). Agarose gel electrophoresis of plasmid DNA from donors and Tox^+ transcipients. a, pXO1::pXO12; b, pXO1 and pXO1 derivatives; c, chromosomal DNA; d, pXO12; e, pBC16. Lanes: 1, Weybridge A UM2-3(pXO1-1, pXO12, pBC16); 2, Weybridge UM44-6(pXO1-2, pXO12, pBC16); 3, Weybridge UM44-8(pXO1-4, pXO12, pBC16); 4, Weybridge A UM23-9(pXO1-3, pBC16); 5, Weybridge A UM23-11(pXO1-5, pXO12, pBC16); 6, Weybridge A UM23-12(pXO1-1::pXO12, pBC16); 7, Weybridge A UM23-13(pXO1-6, pXO12, pBC16); 8, Weybridge A UM17-4(pXO1, pXO12, pBC16), 9, Weybridge A UM23-14(pXO1-7, pXO12, pBC16); 10, Weybridge A UM23-15(pXO1, pXO12, pBC16). (B). Autoradiograph of [^{32}P]-labelled Tn4430 DNA hybridized to the plasmid DNAs shown in A. Lanes 1 to 10 correspond to those shown in A.

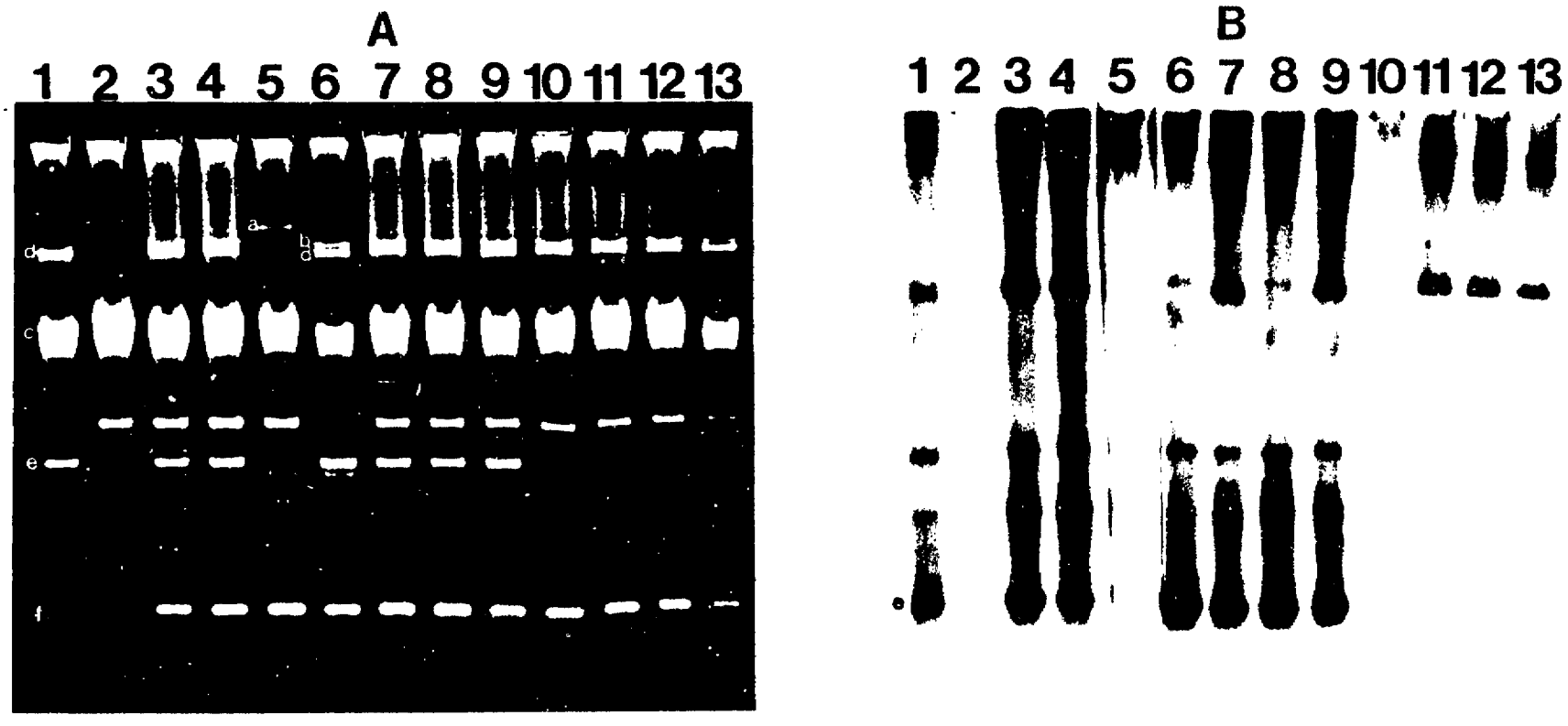

FIG. 2. Demonstration of Tn4430 transposition from pXO12 to pXO2 plasmids following their transfer from B. anthracis to B. cereus 569 by pXO12-mediated conjugation. (A). Agarose gel electrophoresis of plasmid DNA from donors, recipient, and Cap$^+$ transcipients. a, pXO2::pXO12; b, pXO12; c, chromosomal DNA; d, pXO2; e, unnamed nonconjugative plasmid inherited from B. thuringiensis (1); f, pBC16. Lanes: 1, B. anthracis 6602 UM4(pXO2, pXO12[1], pBC16), donor for transcipients in lanes 3 to 5; 2, B. cereus 569 UM20-1, recipient; 3, B. cereus 569 UM20-2(pXO2-2, pXO12, pBC16); 4, B. cereus 569 UM20-3(pXO2-3, pXO12, pBC16); 5, B. cereus 569 UM20-4(pXO2::pXO12, pBC16); 6, B. anthracis 4229 UM12-1(pXO2, pXO12, pBC16), donor for transcipients in lanes 7 to 9; 7, B. cereus 569 UM20-6(pXO2-1, pXO12, pBC16); 8, B. cereus 569 UM20-8(pXO2, pXO12, pBC16); 9, B. cereus 569 UM20-7(pXO2-4, pXO12, pBC16); 10, B. anthracis 4229 UM12-2(pXO2, pXO12, pBC16), donor for transcipients in lanes 11 to 13; 11, B. cereus 569 UM20-9(pXO2-5, pXO12, pBC16); 12, B. cereus 569 UM20-10(pXO2-6, pXO12, pBC16); 13, B. cereus 569 UM20-11(pXO2-7, pXO12, pBC16). (B). Autoradiograph of [^{32}P]-labelled Tn4430 DNA hybridized to the plasmid DNAs shown in A. Lanes 1 to 13 correspond to those shown in A. [1]The pXO12 in 6602 UM4 and transcipients derived from 6602 UM4 is an uncharacterized deletant about tne size of pXO2, and the two did not separate under the conditions used here.

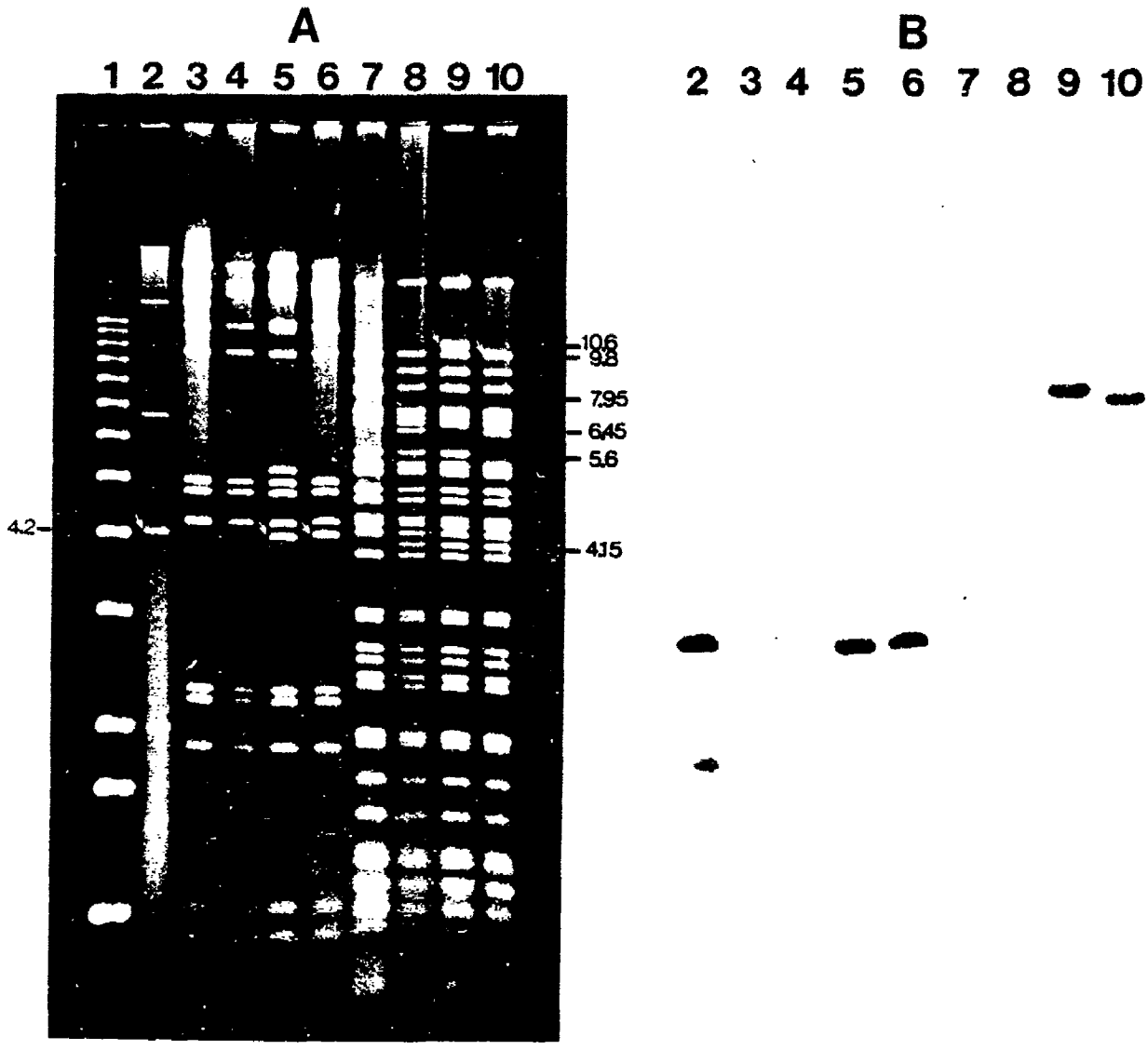

FIG. 3. Agarose gel electrophoresis and corresponding autoradiograph demonstrating insertion of Tn4430 in pXO1-2 and pXO1-3. (A). Agarose gel electrophoresis of plasmid DNAs digested with KpnI and EcoRI. Fragment sizes are given in kilobases. Arrows designate fragments which show homology to probe DNA. Lanes: 1, BRL 1-kb ladder; 2 to 6, pXO12, pXO1, pXO1-1, pXO1-2, and pXO1-3 DNAs, respectively, cut with KpnI; 7 to 10, pXO1, pXO1-1, pXO1-2, and pXO1-3 DNAs, respectively, cut with EcoRI. (B). Autoradiograph of [^{32}P]-labelled Tn4430 DNA hybridized to the restriction digests shown in A. Lanes 2 to 10 correspond to those shown in A.

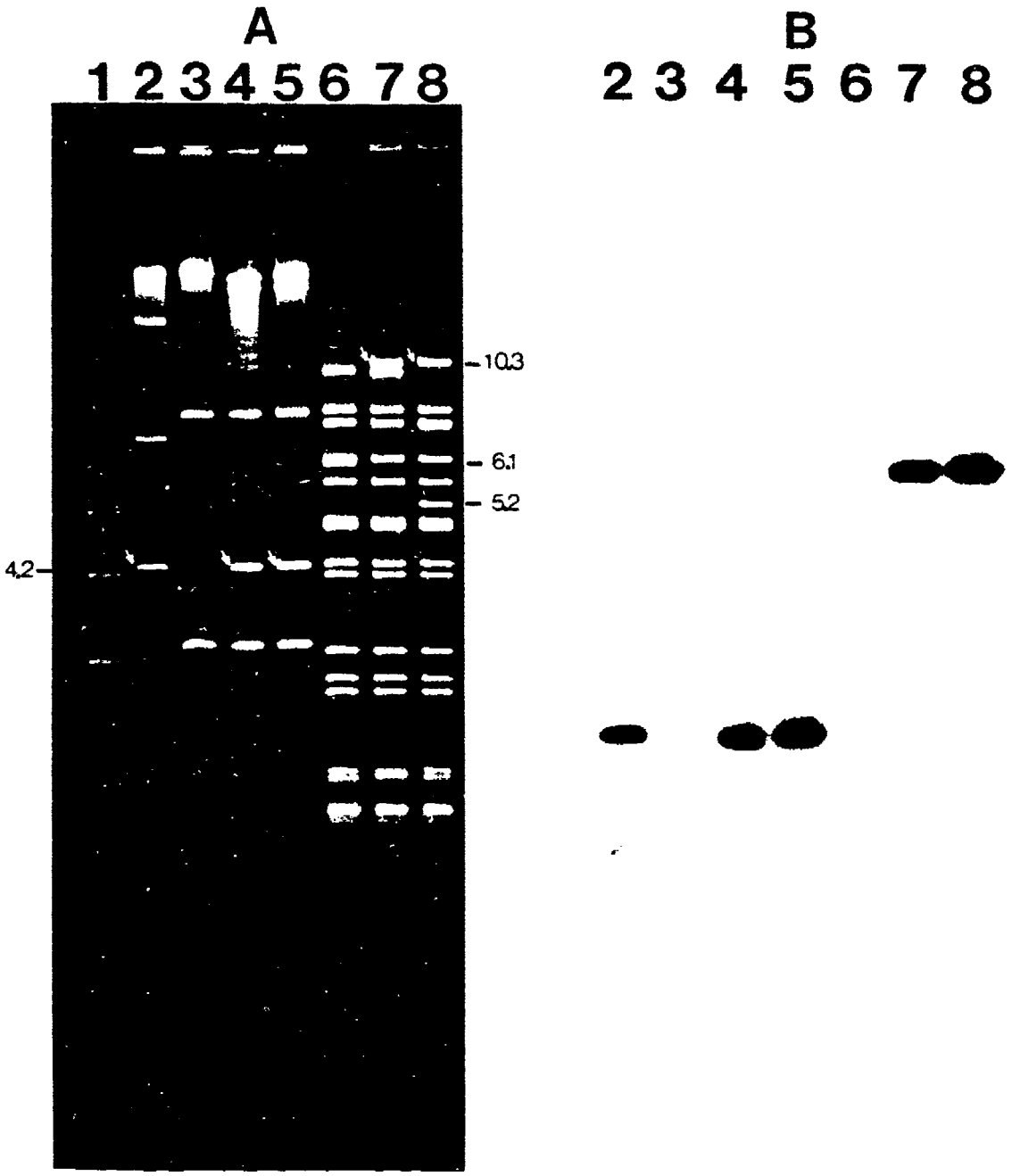

FIG. 4. Agarose gel electrophoresis and corresponding autoradiograph demonstrating insertion of Tn4430 in pXO2-1 and pXO2-2. (A). Agarose gel electrophoresis of plasmid DNAs digested with KpnI and EcoRI. Fragment sizes are given in kilobases. Arrows designate fragments which show homology to probe DNA. Lanes: 1, BRL 1-kb ladder; 2 to 5, pXO12, pXO2, pXO2-1, and pXO2-2 DNAs, respectively, cut with KpnI; 6 to 8, pXO2, pXO2-1, and pXO2-2 DNAs, repectively, cut with EcoRI. (B). Autoradiograph of [^{32}P]-labelled Tn4430 DNA hybridized to the restriction digests shown in A. Lanes 2 to 8 correspond to those shown in A.

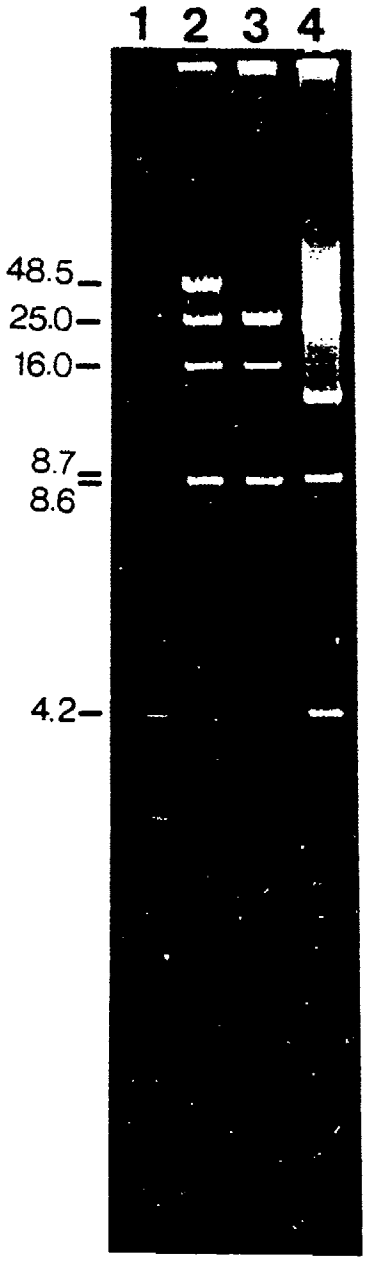

FIG. 5. Agarose gel electrophoresis of AvaI-digested pXO12 DNA and DNA from two Tra$^-$ Cry$^+$ pXO12 deletion derivatives, pXO12-3 and pXO12-4. Fragment sizes are given in kilobases. Lanes: 1, BRL 1-kb ladder; 2, pXO12 DNA; 3, pXO12-3 DNA; 4, pXO12-4 DNA.

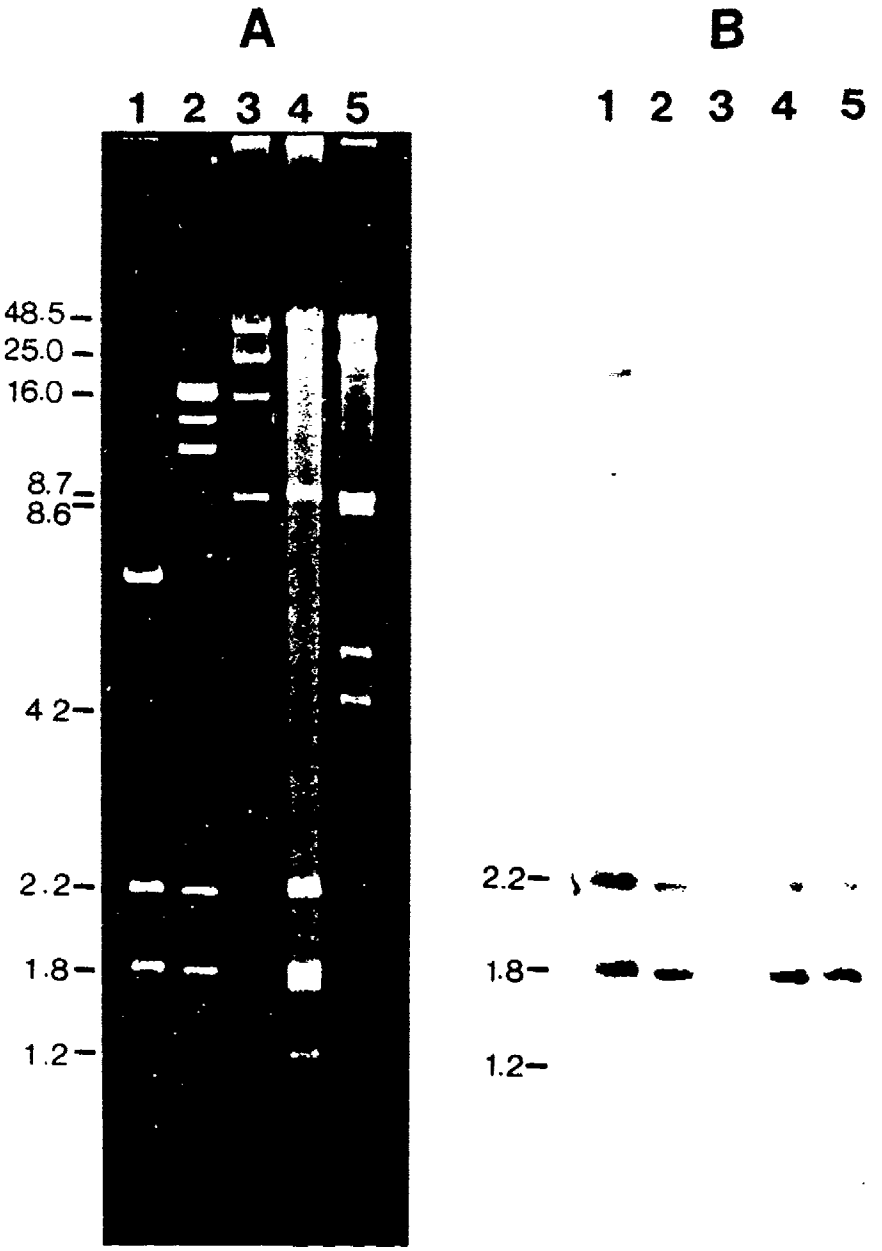

FIG. 6. Agarose gel electrophoresis and corresponding autoradiograph confirming the presence of Tn917 in two Tra$^+$ Cry$^-$ derivatives of pXO12, pXO12-1 and pXO12-2. Fragment sizes are given in kilobases. (A) Agarose gel electrophoresis of plasmid DNAs digested with AvaI. Lanes: 1, pTV1 DNA; 2, pXO503 DNA; 3, pXO12 DNA; 4, pXO12-1 DNA; 5, pXO12-2 DNA. (B) Autoradiograph of ^{32}P-labelled Tn917 DNA hybridized to the restriction digests shown in A. Lanes 1 - 5 correspond to those shown in A.

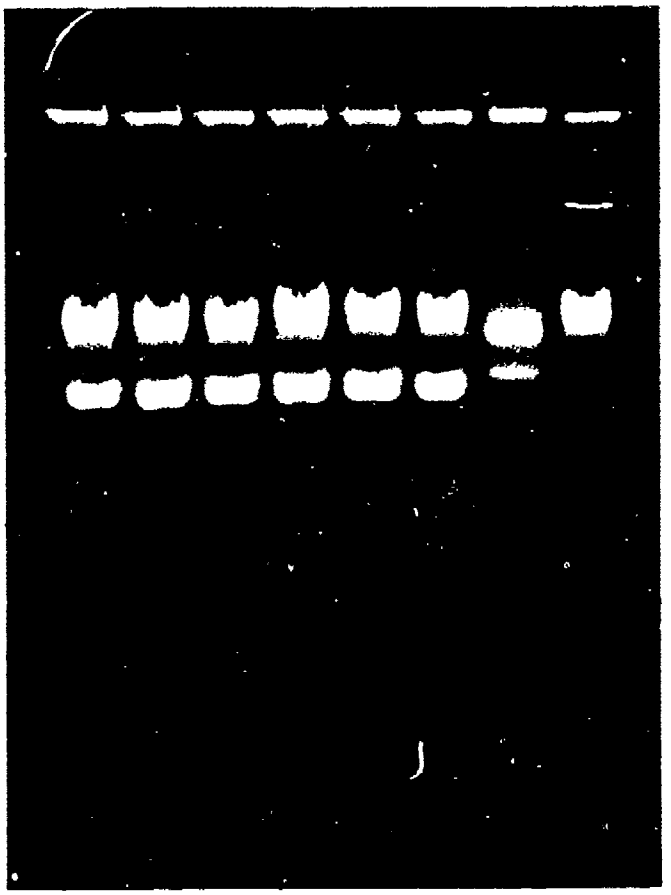

FIG. 7. Agarose gel electrophoresis demonstrating pTV1 in B. anthracis Weybridge A UM23 following transfer of pTV1 from B. cereus 569 by CP-51-mediated transduction. Lanes (from left to right): 1 through 6, Weybridge A UM23, independent transductants containing pTV1; 7, B. subtilis PSL1 UM2(pTV1), the source of pTV1 DNA used to transform B. cereus 569; 8, Weybridge A UM23(pXO1), not transduced. In lanes 1 through 6 the top band is pXO1, the middle band is chromosomal DNA, and the bottom band is pTV1. In lane 7 the top band is chromosomal DNA and the bottom band is pTV1. In lane 8 the top band is pXO1 and the lower band is chromosomal DNA.

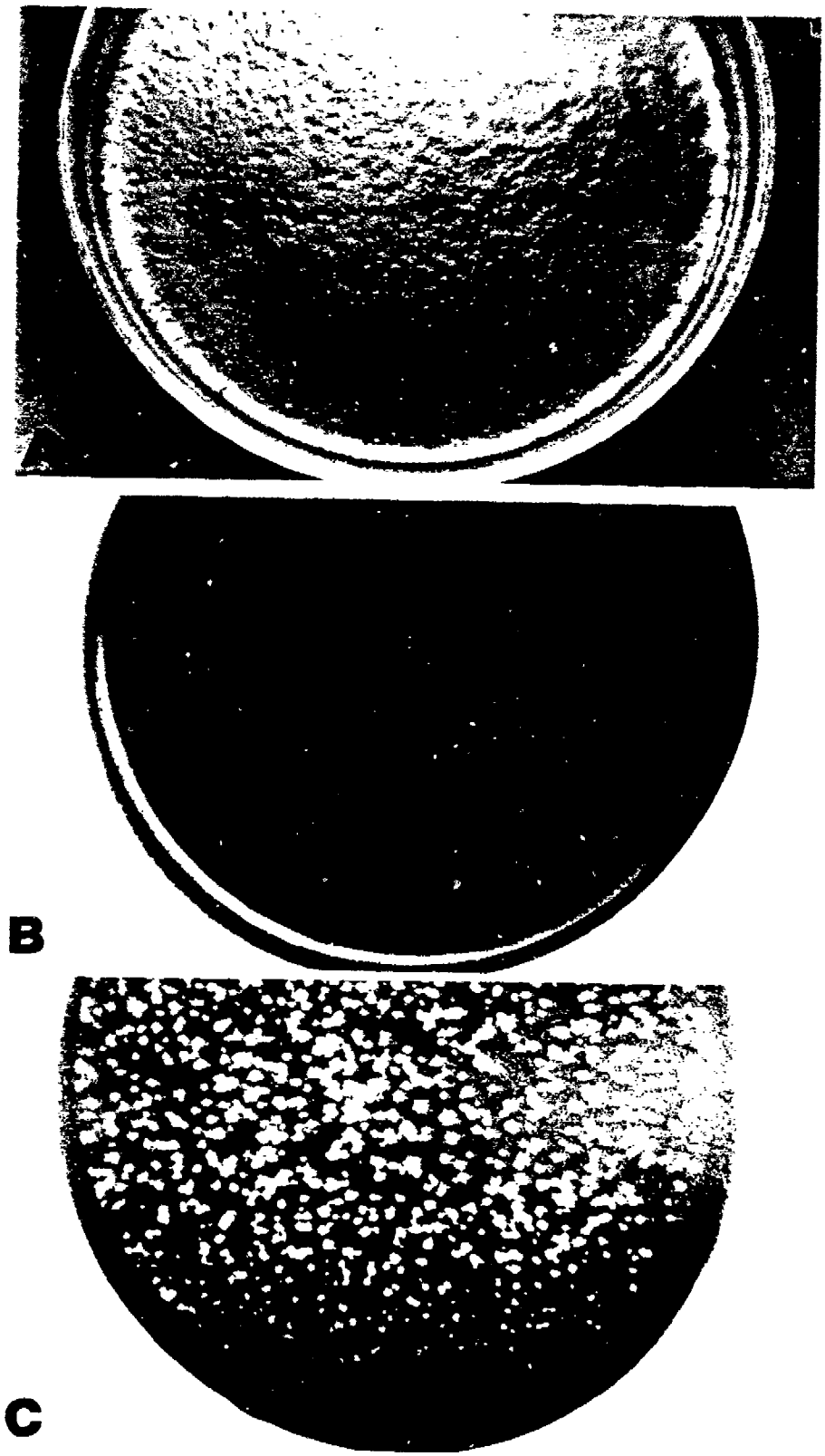

FIG. 8. Phage assay plates: (A) photographed by reflected light; (B) photographed by transmitted light; (C) exposed to chloroform vapor and then photographed by transmitted light.

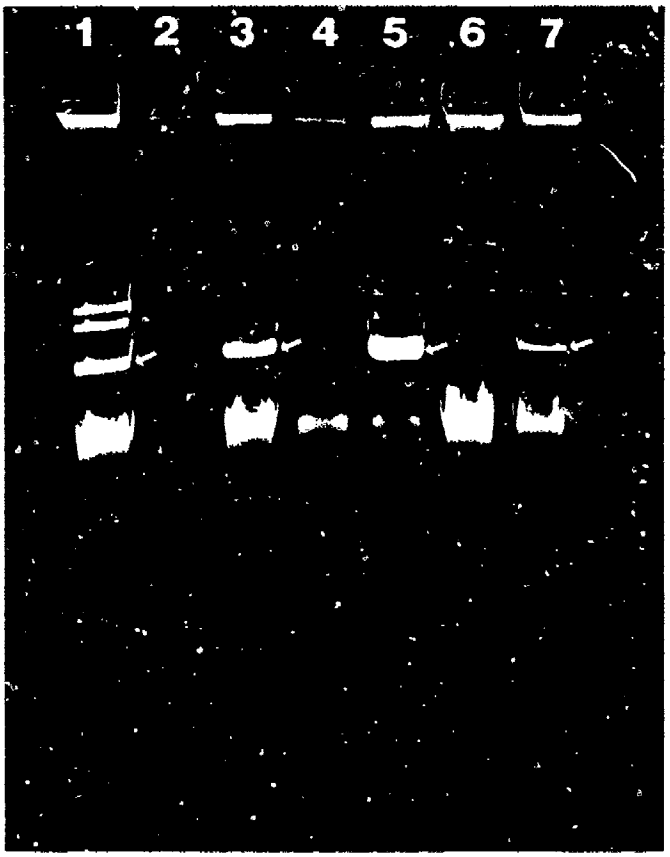

FIG. 9. Agarose gel electrophoresis of plasmid DNA demonstrating the presence of newly acquired plasmid DNA in TP-21 lysogens. Lane 1, B. thuringiensis subsp. kurstaki HD1-9; Lane 2, B. cereus 569; Lane 3, B. cereus 569 (TP-21); Lane 4, B. anthracis 4229R1; Lane 5, B. anthracis 4229R1 (TP-21); Lane 6, B. thuringiensis subsp. toumanoffi 4059; Lane 7, B. thuringiensis subsp. toumanoffi 4059 (TP-21). Arrows point to TP-21 prophage DNA.

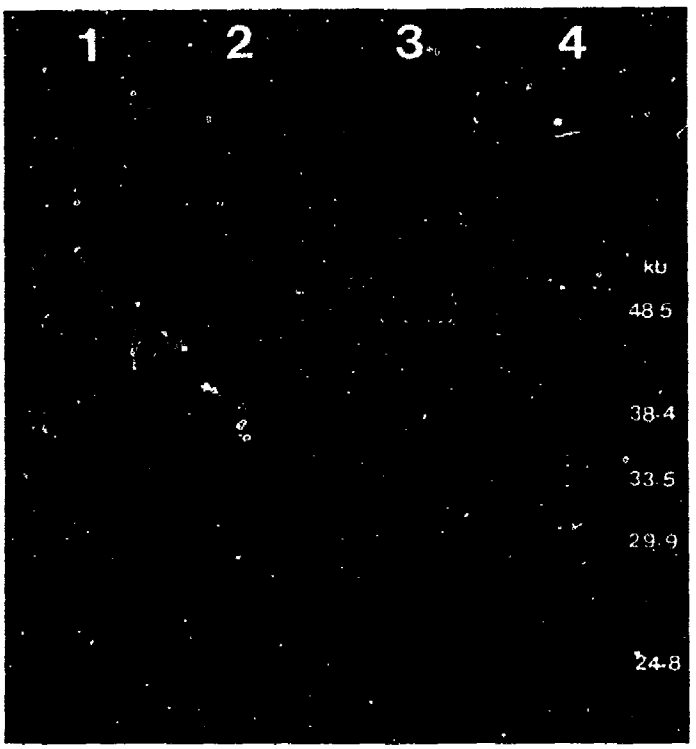

FIG. 10. Agarose gel electrophoresis demonstrating the difference in size of phage and prophage DNA. Lanes 1 and 4, BRL high molecular weight markers; Lane 2, phage DNA; Lane 3, prophage DNA linearized by a KpnI partial digestion.

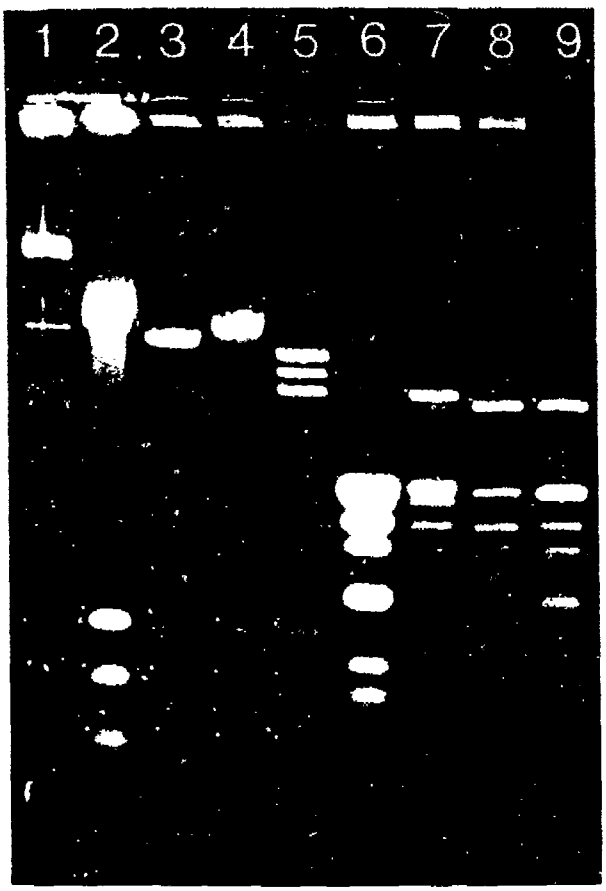

FIG. 11. Agarose gel electrophoresis of AvaI- and EcoRI-digested prophage DNA demonstrating the presence of Tn917. Lanes 1-5, AvaI digests; Lane 1, TP-21; Lane 2, defective TP-21::Tn917-23; Lane 3, defective TP-21::Tn917-13; Lane 4, defective TP-21::Tn917-12; Lane 5, pLS20::Tn917; Lanes 6-9, EcoRI digests; Lane 6, TP-21; Lane 7, defective TP-21::Tn917-23; Lane 8, defective TP-21::Tn917-13; Lane 9, defective TP-21::Tn917-12. Fragments containing Tn917 sequences are marked with an asterisk.

PUBLICATIONS

The following abstracts and papers were published during this reporting period:

1. Heembkerk, D. D., and C. B. Thorne. 1988. Physical and genetic analysis of the Bacillus subtilis (natto) fertility plasmid pLS20. Abstr. Annu. Meet. Am. Soc. Microbiol. H-3.

2. Ruhfel, R. E., and C. B. Thorne. 1988. Physical and genetic characterization of the Bacillus thuringiensis subsp. kurstaki HD-1 extrachromosomal temperate phage TP-21. Abstr. Annu. Meet. Am. Soc. Microbiol. H-4.

3. Koehler, T. M., and C. B. Thorne. 1987. Bacillus subtilis (natto) plasmid pLS20 mediates interspecies plasmid transfer. J. Bacteriol. **169**:5271-5278.

4. Reddy, A., L. Battisti, and C. B. Thorne. 1987. Identification of self-transmissible plasmids in four Bacillus thuringiensis subspecies. J. Bacteriol. **169**:5263-5270.

The following paper has been submitted to J. Bacteriol. for publication:

Green, B. D., L. Battisti, and C. B. Thorne. Involvement of Tn4430 in the transfer of Bacillus anthracis plasmids mediated by the Bacillus thuringiensis plasmid pXO12.

The following Ph. D. dissertations were written on research carried out under this contract:

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1. Koehler, Theresa, M. Plasmid-related differences in capsule production by Bacillus anthracis and Characterization of a fertility plasmid from Bacillus subtilis (natto). Ph. D. Dissertion. University of Massachusetts, Amherst. September 1987.

2. Battisti, Lauren A. Characterization of two self-transmissible plasmids from a strain of Bacillus thuringiensis subsp. thuringiensis. Ph. D. Dissertation. University of Massachusetts, Amherst. February 1987.

3. Green, Brian D. Mobilization of the Bacillus anthracis plasmids pXO1 and pXO2 by the Bacillus thuringiensis fertility plasmid pXO12. Ph. D. Dissertation. University of Massachusetts, Amherst. February 1988.

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1. **Battisti, L., B. D. Green, and C. B. Thorne.** 1985. Mating system for the transfer of plasmids among Bacillus anthracis, Bacillus cereus and Bacillus thuringiensis. J. Bacteriol. **162**:543-550.
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5. **Clark, A. J., and G. J. Warren.** 1979. Conjugal transmission of plasmids. Annu. Rev. Genet. **13**:99-125.
6. **Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in Bacillus anthracis. Infect. Immun. **49**:291-297.
7. **Heierson, A., R. Landen, A. Lovgren, G. Dalhammar, and H. B. Boman.** 1987. Transformation of vegetative cells of Bacillus thuringiensis by plasmid DNA. J. Bacteriol. **29**:1147-1152.
8. **Iida, S., and W. Arber.** 1979. Multiple physical differences in the genome structure of functionally related bacteriophages P1 and P7. Mol. Gen. Genet. **173**:249-261.
9. **Kado, C. I., and S. -T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1356-1373.
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